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VIRUS-CELL INTERACTIONS

Hepatitis C Virus Escape Studies of Human Antibody AR3A Reveal a High Barrier to Resistance and Novel Insights on Viral Antibody Evasion Mechanisms

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ABSTRACT Yearly, ~2 million people become hepatitis C virus (HCV) infected, resulting in an elevated lifetime risk for severe liver-related chronic illnesses. Characterizing epitopes of broadly neutralizing antibodies (NAbs), such as AR3A, is critical to guide vaccine development. Previously identified alanine substitutions that can reduce AR3A binding to expressed H77 envelope were introduced into chimeric cell culture-infectious HCV recombinants (HCVcc) H77(core-NS2)/JFH1. Substitutions G523A, G530A, and D535A greatly reduced fitness, and S424A, P525A, and N540A, although viable, conferred only low-level AR3A resistance. Using highly NAb-sensitive hypervariable region 1 (HVR1)-deleted HCVcc, H77/JFH1$_{\Delta HVR1}$ and J6(core-NS2)/JFH1$_{\Delta HVR1}$, we previously reported a low barrier to developing AR5A NAb resistance substitutions. Here, we cultured Huh7.5 cells infected with H77/JFH1, H77/JFH1$_{\Delta HVR1}$, or J6/JFH1$_{\Delta HVR1}$ with AR3A. We identified the resistance envelope substitutions M345T in H77/JFH1, L438S and F442Y in H77/JFH1$_{\Delta HVR1}$, and D431G in J6/JFH1$_{\Delta HVR1}$. M345T increased infectivity and conferred low-level AR3A resistance to H77/JFH1 but not H77/JFH1$_{\Delta HVR1}$. L438S and F442Y conferred high-level AR3A resistance to H77/JFH1$_{\Delta HVR1}$ but abrogated the infectivity of H77/JFH1. D431G conferred AR3A resistance to J6/JFH1$_{\Delta HVR1}$ but not J6/JFH1. This was possibly because D431G conferred broadly increased neutralization sensitivity to J6/JFH1$_{D431G}$ but not J6/JFH1$_{\Delta HVR1/D431G}$ while decreasing scavenger receptor class B type I coreceptor dependency. Common substitutions at positions 431 and 442 did not confer high-level resistance in other genotype 2a recombinants [JFH1 or T9(core-NS2)/JFH1]. Although the data indicate that AR3A has a high barrier to resistance, our approach permitted identification of low-level resistance substitutions. Also, the HVR1-dependent effects on AR3A resistance substitutions suggest a complex role of HVR1 in virus escape and receptor usage, with important implications for HCV vaccine development.

IMPORTANCE Hepatitis C virus (HCV) is a leading cause of liver-related mortality, and limited treatment accessibility makes vaccine development a high priority. The vaccine-relevant cross-genotype-reactive antibody AR3A has shown high potency, but the ability of the virus to rapidly escape by mutating the AR3A epitope (barrier to resistance) remains unexplored. Here, we succeeded in inducing only low-level AR3A resistance, indicating a higher barrier to resistance than what we have previously reported for AR5A. Furthermore, we identify AR3A resistance substitutions that have hypervariable region 1 (HVR1)-dependent effects on HCV viability and on broad neutralization sensitivity. One of these substitutions increased envelope breathing and decreased scavenger receptor class B type I HCV coreceptor dependency, both in an HVR1-dependent fashion. Thus, we identify novel AR3A-specific resistance substitutions and the role of HVR1 in protecting HCV from AR3-targeting antibodies.

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These viral escape mechanisms should be taken into consideration in future HCV vaccine development.

KEYWORDS HVR1, antibody escape, hepatitis C virus, immune evasion, liver disease, vaccine

Hepatitis C virus (HCV) is a major cause of chronic liver diseases worldwide. Acute HCV infection, seen in about 2 million people each year, often progresses to chronic hepatitis, and it is estimated that up to 150 million people have chronic HCV infection, with approximately 400,000 people dying every year from HCV-related liver illnesses, including cirrhosis and hepatocellular carcinoma (1, 2). The availability of interferon-free direct-acting antiviral (DAA) therapies offers cure rates of >95% (2, 3), but the high cost of treatment limits their use, and treatment does not protect against reinfection. Moreover, it is estimated that at least 80% of all HCV infections are undiagnosed (4–6). Thus, a vaccine is needed to globally eradicate this pervasive human pathogen (7).

HCV belongs to the *Flaviviridae* family and is divided into 6 clinically important genotypes (1, 8, 9). HCV is an enveloped positive-stranded RNA virus, and its genome encodes a single polyprotein that is processed into 3 structural proteins (core, E1, and E2), p7, and 6 nonstructural proteins (NS2 to NS5B). The envelope protein complex E1/E2 is the principal target of neutralizing antibodies (NAbs) and is therefore of key interest in the development of HCV vaccine candidates (10). NAbs have been associated with lower levels of acute-phase viremia in patients and in chimpanzees as well as with clearance of infection in patients and in human liver-chimeric mice (11–15). In addition, passive immunization of chimpanzees and human liver-chimeric mice by infusion with NAbs was shown to protect against HCV infection (16–19). However, an effective HCV vaccine has to overcome the high genetic diversity of HCV (20), which will require the identification of cross-genotype conserved epitopes with high barriers to resistance (i.e., difficult for the virus to develop high-level [>10-fold] resistance without compromising fitness).

We have characterized NAbs of human origin against five different antigenic regions (termed AR1 to AR5) on the E1/E2 glycoprotein complex (21, 22). Three of these antibodies, AR3A, AR4A, and AR5A, target epitopes that are conserved across genotypes (21–23). However, high epitope conservation does not necessarily result in a high barrier to resistance, as we recently reported for AR5A, for which the virus rapidly acquired AR5A resistance substitutions when cultured with the antibody (24). AR3A has been shown to provide protection *in vivo* when tested in a mouse model (14, 21), underscoring the importance of testing the barrier to resistance for this antibody.

Selection of virus escape mutants in HCV cell culture (HCVcc) has been shown to be an effective methodology to identify epitope-specific mutations *in vitro*, and some of these have been shown to have *in vivo* relevance (24–30). However, viral escape mutants are generally difficult to generate with HCVcc because of the inherently high antibody resistance of most HCV isolates. In addition, we have shown that the high fitness of certain viruses, like core-NS2 recombinants J6/JFH1 and SA13/JFH1, permits the viruses to spread in culture, even at high concentrations of antibody, without developing resistance substitutions (24, 31), possibly aided by high levels of cell-to-cell spread (32, 33). Previously, we studied AR5A resistance by using novel HCVcc with a deletion of hypervariable region 1 (HVR1) (24). HVR1 had been shown to be dispensable for fitness *in vitro* and *in vivo* (16, 34–36), with a possible involvement in interactions with the two HCV entry coreceptors scavenger receptor class B type I (SR-BI) and low-density lipoprotein receptor (37, 38). In addition, the removal of HVR1 confers greatly increased sensitivity of HCV to NAbs (23, 34, 36). In a previous study, we cultured HVR1-deleted viruses H77/JFH1$_{\Delta HVR1}$ (genotype 1a) and J6/JFH1$_{\Delta HVR1}$ (genotype 2a) in the presence of AR5A to induce and characterize pangenotypic escape substitutions (24). The generally low barrier to resistance against AR5A (defined as rapid induction of

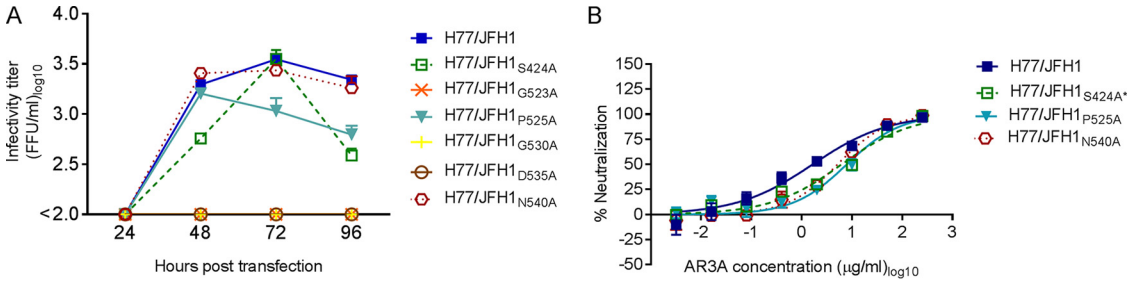

FIG 1 Envelope substitutions identified by alanine scanning for AR3A reactivity at the protein level have various effects on H77/JFH1 virus infectivity. (A) Huh7.5 cells were transfected with *in vitro*-transcribed RNA of the indicated recombinants. Supernatants were collected at the indicated time points, and HCV infectivity titers were determined. The lower level of quantification was 100 FFU/ml. (B) First passages of the indicated viruses were subjected to a dilution series of antibody AR3A from 250 μg/ml to 0.0032 μg/ml. The virus-antibody mixes along with virus only were added to Huh7.5 cells for 4 h prior to washing and addition of fresh medium. Following a total of 48 h of infection, the cells were immunostained, and the number of FFU per well was counted as described in Materials and Methods. Error bars represent standard errors of the means (SEM) of data from 4 replicates normalized to values from 8 replicates of virus only. The data were analyzed using four-parameter curve fitting to obtain a sigmoidal dose-response curve. *, the virus had the additional substitutions Y361H (T1422C) and N415S (A1585G).

high-level AR5A resistance for both studied isolates) indicated that this epitope may not be an optimal target in future rational vaccine development.

Due to the potential of a conserved epitope targeted by the human monoclonal NAb AR3A in HCV vaccine development, we here assess the AR3A barrier to resistance. We first used previously determined alanine scanning mutagenesis data to study substitutions resulting in reduced AR3A binding and found that the viable substitutions conferred only low-level (<10-fold) AR3A resistance. By culturing H77/JFH1 with and without HVR1 in the presence of AR3A, we identified the epitope-specific escape substitutions M345T, L438S, and F442Y. In a similar approach for HVR1-deleted J6/JFH1, we identified the escape substitution D431G. The effects of these substitutions on fitness and broad NAb sensitivity depended on the presence of HVR1. For D431G, we observed decreased dependence on the HCV entry factor SR-BI and a broad increase in NAb sensitivity, which was linked to increased virus breathing. Common polymorphisms at positions 431 and 442 frequently resulted in fitness loss and did not confer high-level AR3A resistance, suggesting that the epitope has a high barrier to escape. In addition, our data suggest that HVR1-mediated protection is linked to virus breathing and SR-BI usage, with important implications for understanding HCV immune evasion.

RESULTS

Alanine scanning substitutions previously shown to affect AR3A binding to H77 E1/E2 have various effects on viral fitness of H77/JFH1. The E2 alanine substitutions S424A, G523A, P525A, G530A, D535A, and N540A were previously shown to greatly reduce AR3A antibody binding to H77 E1/E2 (21). Here, the alanine substitutions were introduced into the cell culture-infectious recombinant H77/JFH1, which harbors core-NS2 from H77 (39). In transfections of Huh7.5 cells, we observed that substitution N540A did not impact virus infectivity, S424A and P525A decreased virus fitness, and G523A, G530A, and D535A resulted in titers below the detection limit (Fig. 1A). We passaged H77/JFH1$_{S424A}$, H77/JFH1$_{P525A}$, and H77/JFH1$_{N540A}$ and sequenced the envelope genes of these first-passage virus stocks. We found that H77/JFH1$_{S424A}$ had the additional substitutions Y361H (E1; T1422C) and N415S (E2; A1585G) (all positions in this study are given relative to the H77 reference genome [GenBank accession number AF009606]). The H77/JFH1$_{P525A}$ and H77/JFH1$_{N540A}$ viruses maintained the alanine substitutions without additional changes. By testing neutralization sensitivity, we determined the AR3A 50% inhibitory concentration (IC_{50}) against these viruses. The viruses with alanine substitutions (H77/JFH1$_{S424A}$ IC_{50} = 6.2 μg/ml; H77/JFH1$_{P525A}$ IC_{50} = 7.8 μg/ml; H77/JFH1$_{N540A}$ IC_{50} = 5.1 μg/ml) were only ~3- to 5-fold more resistant than the parental virus (IC_{50} = 1.6 μg/ml) (Fig. 1B).

Culturing H77/JFH1 in the presence of AR3A results in escape and low-level antibody resistance. H77/JFH1-infected Huh7.5 cells were split into separate wells and

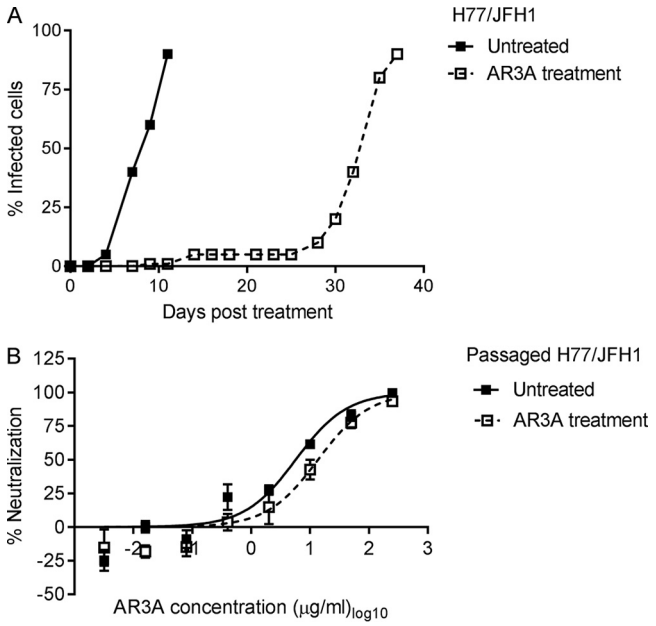

FIG 2 Culture of H77/JFH1 retaining HVR1 in the presence of NAb AR3A results in low-level resistance. (A) Huh7.5 cells were infected with H77/JFH1 and either treated with 200 μg/ml of AR3A antibody or not treated (control) during the indicated period of time. (B) First passages of the virus supernatants taken from treatments and untreated controls were subjected to a dilution series of AR3A from 250 μg/ml to 0.0032 μg/ml, and neutralization was assessed and analyzed as described in the legend of Fig. 1, except that we used three-parameter curve fitting to obtain a sigmoidal dose-response curve.

either treated with 200 μg/ml of AR3A or left untreated (Fig. 2A and Table 1). AR3A delayed the spread of H77/JFH1 by more than 20 days. An antibody-free virus stock of the treated virus was generated to test AR3A neutralization susceptibility (Fig. 2B and Table 1). The treated virus (IC_{50} = 14 μg/ml) was ~3 times more resistant than the parental virus (IC_{50} = 5.7 μg/ml). This was confirmed in an independent experiment (not shown). By sequencing the envelope from recovered HCV RNA, we identified the E1 substitution M345T (E1; T1375C) (Table 1).

M345T increases fitness of H77/JFH1 and confers low-level AR3A-specific antibody resistance. The substitution M345T (E1) was introduced into H77/JFH1 and H77/JFH1$_{\Delta HVR1/N417D/N532D}$; an H77/JFH1$_{\Delta HVR1}$ variant with fitness comparable to that of H77/JFH1 (24). Transfecting Huh7.5 cells with these recombinants revealed that M345T increased the fitness of H77/JFH1 (Fig. 3A) but decreased the fitness of H77/JFH1$_{\Delta HVR1/N417D/N532D}$ (Fig. 3B). Virus supernatants from the peak of infection were used to generate first-passage stocks, and their envelope sequences were confirmed. By comparing AR3A neutralization susceptibilities of the viruses, we found that H77/JFH1$_{M345T}$ (IC_{50} = 8.1 μg/ml) was ~5-fold less susceptible to AR3A neutralization than H77/JFH1 (IC_{50} = 1.5 μg/ml) (Fig. 3C). In contrast, AR3A susceptibilities were similar when comparing H77/JFH1$_{\Delta HVR1/N417D/N532D/M345T}$ (IC_{50} = 0.00010 μg/ml) with H77/JFH1$_{\Delta HVR1/N417D/N532D}$ (IC_{50} = 0.00022 μg/ml) (Fig. 3D). Finally, to investigate whether M345T had a broad effect on neutralization sensitivity, we compared AR4A (Fig. 3E and F) and AR5A (Fig. 3G and H) susceptibilities of the viruses. The substitution M345T did not affect susceptibility to AR4A and AR5A, suggesting that M345T caused low-level, but AR3A-specific, resistance.

Culturing H77/JFH1$_{\Delta HVR1}$ in the presence of AR3A results in escape and high-level antibody resistance. Since the induction of escape variants for H77/JFH1 resulted in only low-level resistance by the substitution M345T, we instead attempted to isolate resistant variants by culturing Huh7.5 cells infected with highly NAb-sensitive H77/JFH1$_{\Delta HVR1}$ in the presence of AR3A. Three cultures were treated with 5 μg/ml of AR3A (treatments I, II, and III) (Fig. 4A and Table 1), and one was left untreated. In all

TABLE 1 Coding mutations observed in the HCV envelope proteins following AR3A treatment[c]

Parameter	AR3A (μg/ml)	AR3A resistance level (IC_{50} treated/IC_{50} untreated)	Days posttreatment[b]	Value for HCV gene									
				E1	E1	E2	E2	E2	E2	E2	E2	E2	E2
Genome position				1170	1374	1632	1643	1653	1652	1665	1671	1935	2069
H77 reference (GenBank accession no. AF009606) position				1171	1375	1633	1644	1654	1653	1666	1672	1936	2070
Nucleotide				T	T	A	A	T	A	T	A	A	A
H77/JFH1													
Treatment I	200	~3	37		C								
$H77/JFH1_{\Delta HVR1}$													
Treatment I	5	~40	42							A	G	C	C
Treatment II	5	~100	47							A	G	C	C
Treatment III	5	~300	75				C/A			A		C	C
$H77/JFH1b_{\Delta HVR1/N417D/N532D}$													
Treatment I	20	~250,000	56					C			G		
Treatment II	20	~300,000	89					C			G		
$J6/JFH1_{\Delta HVR1}$													
Treatment I	40	~9100	44	C		G							
Treatment II	40	~1400	137	C		G			G/A				
Amino acid no. and type													
Polyprotein position				277	345	431	435	438	438	442	444	532	577
H77 reference (GenBank accession no. AF009606) position				277	345	431	435	438	438	442	444	532	577
Amino acid change				V > A	M > T	D > G	T > P	L > S	I > V	F > Y	Q > R	N > T	N > H
Amino acid observed at least twice in Los Alamos database[a]													
Within genotypes				VI	MFVL	DE	TAPS	LVFIM	LIM	FLIMV	QRHYSAFLTVNK	NDK	NDKSRGELHR
Across genotypes (unless also within genotypes)				GME	IA	ASNT			VF				AVQ

[a]Data were taken from the Los Alamos database on 21 August 2017.

[b]The numbers represent the total number of days that the virus infection was treated with AR3A before spreading to at least 80% of cells.

[c]Mutations were identified by direct sequence analysis of HCV RNA amplified by reverse transcription-PCR (RT-PCR) from viruses recovered in virus passages of the indicated treatments. Shaded columns denote mutations observed to confer resistance against the NAb AR3A.

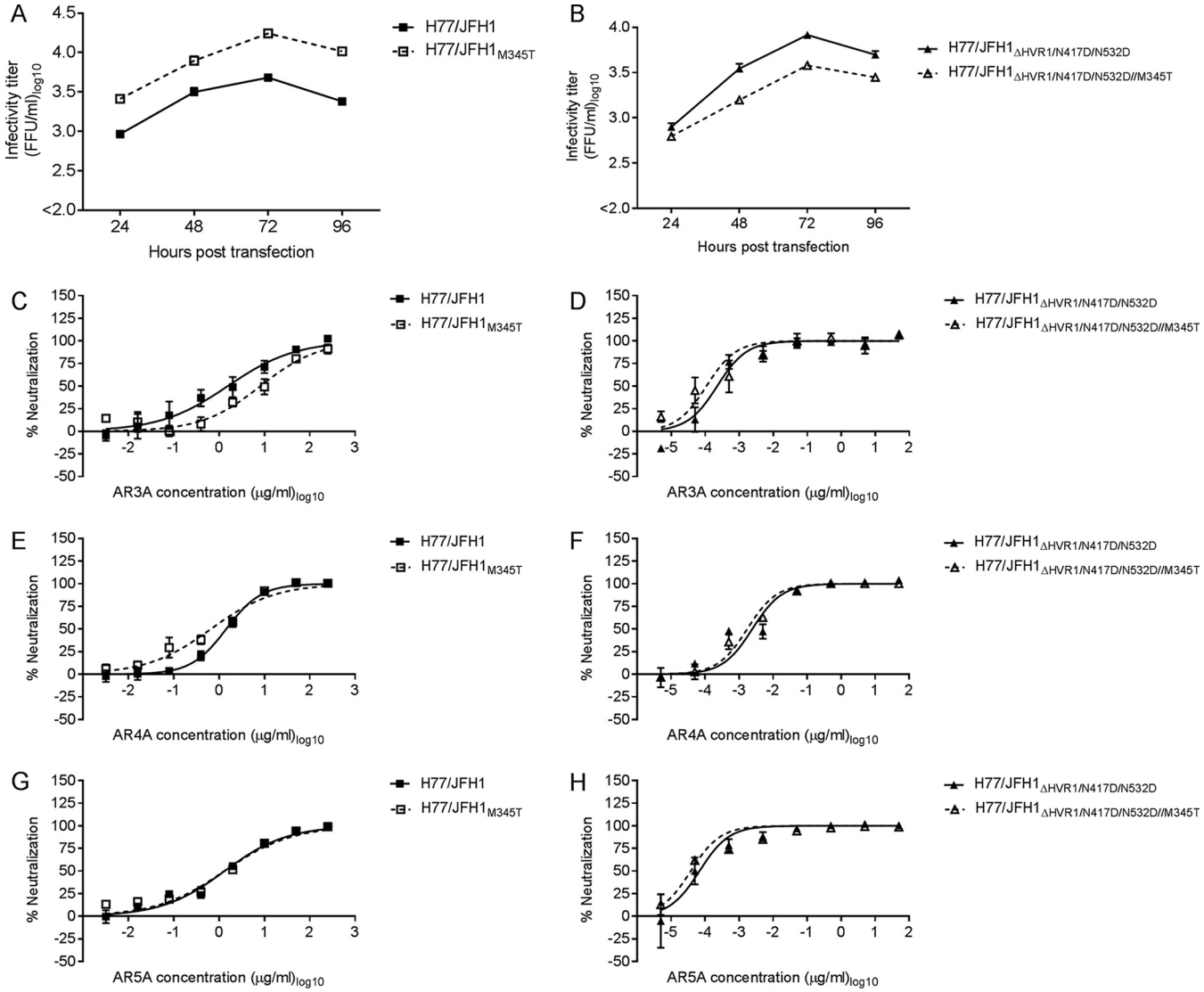

FIG 3 The E1 substitution M345T increases virus fitness and confers low-level AR3A-specific resistance to H77/JFH1 but not to H77/JFH1$_{\Delta HVR1/N417D/N532D}$. (A and B) Huh7.5 cells were transfected with *in vitro*-transcribed RNA of the indicated recombinants with (A) or without (B) HVR1. Supernatants were collected at the indicated time points, and HCV infectivity titers were determined. The lower level of quantification was 100 FFU/ml. The effects of M345T on virus fitness were confirmed in an independent transfection. (C to H) First passages of the viruses were subjected to a dilution series of AR3A, AR4A, and AR5A from 250 μg/ml to 0.0032 μg/ml (C, E, and G) or 50 μg/ml to 0.000005 μg/ml (D, F, and H), and neutralization was assessed and analyzed as described in the legend of Fig. 1, except that we used three- or four-parameter curve fitting to obtain a sigmoidal dose-response curve.

treated cultures, the number of infected cells decreased after day 5 posttreatment. Infection in treatment I started to spread at day 33 posttreatment and reached 80% infected cells at day 42. Infection in treatment II started to spread at day 38 posttreatment and reached 80% infected cells at day 47. Finally, infection in treatment III started to spread at day 59 posttreatment and reached 80% infected cells at day 75. Cell supernatants were collected from the untreated control and from all treated cultures once they reached 80% infected cells.

The cell supernatants were used to generate first-passage virus stocks for AR3A sensitivity testing. The AR3A-treated viruses were ~100-fold less sensitive against AR3A neutralization (treatment I, IC_{50} = 0.39 μg/ml; treatment II, IC_{50} = 1.0 μg/ml; treatment III, IC_{50} = 2.9 μg/ml; untreated, IC_{50} = 0.0096 μg/ml) (Fig. 4B and Table 1). Sequence analysis of E1 and E2 of virus genomes recovered from treatments I, II, and III identified the common substitutions F442Y (T1666A), N532T (A1936C), and N577H (A2070C) (Table 1). The presence of these E2 substitutions in all three cases strongly indicated that one or more of them were causing the observed AR3A antibody resistance.

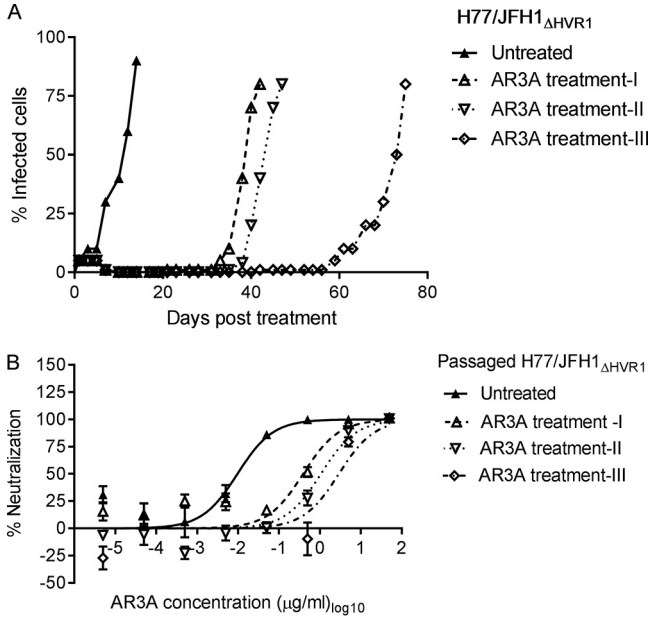

FIG 4 Culturing H77/JFH1$_{\Delta HVR1}$ in the presence of NAb AR3A results in high-level resistance. (A) Huh7.5 cells were infected with H77/JFH1$_{\Delta HVR1}$ and treated with 5 μg/ml of AR3A antibody during the indicated period of time. Treatments I, II, and III were performed independently and cultured along with the untreated control. (B) First passages of virus supernatants taken from treatments I, II, and III and the untreated control were subjected to a dilution series of AR3A from 50 μg/ml to 0.000005 μg/ml, and neutralization was assessed and analyzed as described in the legend of Fig. 1, except that we used three-parameter curve fitting to obtain a sigmoidal dose-response curve.

F442Y confers high-level AR3A resistance to H77/JFH1$_{\Delta HVR1}$ but abrogates infectivity of H77/JFH1. We introduced F442Y, N532T, and N577H alone or in combination into H77/JFH1$_{\Delta HVR1}$ to test which of these substitutions conferred AR3A resistance. By transfecting Huh7.5 cells with these recombinants, we observed that N577H alone did not affect virus titers (Fig. 5A), whereas F442Y alone or combined with N577H reduced the fitness of H77/JFH1$_{\Delta HVR1}$. However, the viruses with both F442Y and N532T reached virus titers comparable to those of the parental virus (Fig. 5B). Finally, viruses with N532T alone (Fig. 5A) or in combination with N577H (Fig. 5B) reached higher titers than the parental virus, suggesting that N532T was a nonspecific cell culture-adaptive substitution.

We generated a first-passage stock of every virus and sequenced the envelope genes. Most envelope sequences were genetically stable in the first passage. However, the substitution F442Y reverted to phenylalanine for H77/JFH1$_{\Delta HVR1/F442Y}$ and H77/JFH1$_{\Delta HVR1/F442Y/N577H}$, probably due to low viral fitness. By testing AR3A neutralization sensitivity of the viruses, we found that H77/JFH1$_{\Delta HVR1/F442Y/N532T}$ (IC_{50} = 12 μg/ml) and H77/JFH1$_{\Delta HVR1/F442Y/N532T/N577H}$ (IC_{50} = 3.0 μg/ml) were ~10,000-fold less susceptible to AR3A neutralization than H77/JFH1$_{\Delta HVR1}$ (IC_{50} = 0.00070 μg/ml) (Fig. 5C and D). In addition, N532T alone or in combination with N577H increased virus susceptibility ~10-fold against AR3A neutralization (H77/JFH1$_{\Delta HVR1/N532T}$ IC_{50} = 0.000061 μg/ml; H77/JFH1$_{\Delta HVR1/N532T/N577H}$ IC_{50} = 0.000091 μg/ml). Thus, we found that the E2 substitution F442Y decreased AR3A neutralization susceptibility of H77/JFH1$_{\Delta HVR1}$ and that adaptive E2 substitutions were required to offset the fitness loss caused by F442Y.

The E2 substitutions F442Y and N532T were introduced alone or in combination into the parental recombinant H77/JFH1. By transfecting Huh7.5 cells with these recombinants, we observed that N532T had a slight negative effect on virus fitness (Fig. 5E). F442Y alone or in combination with N532T did not spread to more than 20% of cells at day 4 posttransfection, and virus infectivity could not be detected at any time point in three independent experiments. Thus, F442Y was nonviable in H77/JFH1.

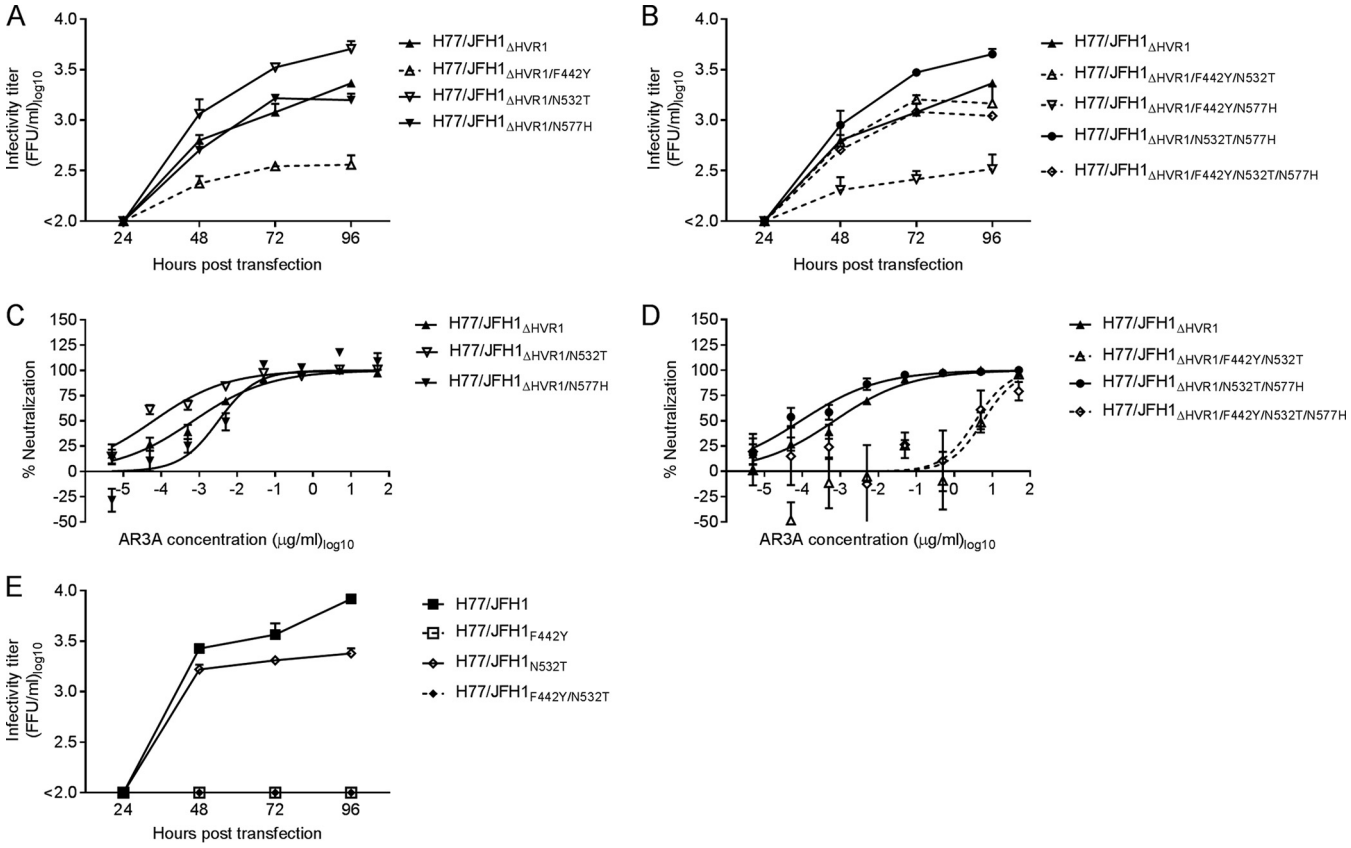

FIG 5 The substitution F442Y confers AR3A resistance to H77/JFH1$_{\Delta HVR1}$ and abrogates virus infectivity of H77/JFH1 retaining HVR1. (A, B, and E) Huh7.5 cells were transfected with *in vitro*-transcribed RNA of the indicated HVR1-deleted recombinants with single (A) or combined (B) substitutions and recombinants retaining HVR1 (E). Supernatants were collected at the indicated time points, and HCV infectivity titers were determined. The lower level of quantification was 100 FFU/ml. (C and D) First passages of the indicated viruses with single (C) or combined (D) substitutions were subjected to a dilution series of AR3A from 50 μg/ml to 0.000005 μg/ml, and neutralization was assessed and analyzed as described in the legend of Fig. 1.

Different AR3A escape substitutions are selected using H77/JFH1$_{\Delta HVR1/N417D/N532D}$. In another attempt to identify viable H77/JFH1 escape substitutions against AR3A, we treated two cultures of Huh7.5 cells infected with H77/JFH1$_{\Delta HVR1/N417D/N532D}$ with 20 μg/ml of AR3A (treatments I and II) (Fig. 6A and Table 1), and one well was left untreated. The infection of cells from treatment I started to spread at day 31 posttreatment and reached 80% infection at day 56. Infection of cells from treatment II started to spread at day 49 posttreatment and reached 80% infection at day 89.

Virus stocks were made from supernatants of control and treated cultures. These were used to test AR3A sensitivity (Fig. 6B and Table 1). The AR3A-treated viruses (treatment I, IC_{50} = 38 μg/ml; treatment II, IC_{50} > 50 μg/ml) were ~250,000-fold less sensitive than the untreated virus (IC_{50} = 0.00015 μg/ml). Sequencing the envelope genes revealed that treatments I and II shared the E2 substitutions L438S (T1654C) and Q444R (A1672G) (Table 1).

L438S confers high-level AR3A resistance in H77/JFH1$_{\Delta HVR1/N417D/N532D}$ but abrogates infectivity of H77/JFH1. To identify which E2 substitutions conferred AR3A resistance, we introduced L438S and Q444R alone or in combination in H77/JFH1$_{\Delta HVR1/N417D/N532D}$. By transfecting Huh7.5 cells with these recombinants, we observed that while Q444R did not impact fitness by itself, it restored the infectivity loss caused by L438S (Fig. 7A). Next, we generated first-passage virus stocks of H77/JFH1$_{\Delta HVR1/N417D/N532D/Q444R}$ and H77/JFH1$_{\Delta HVR1/N417D/N532D/L438S/Q444R}$. Sequencing of the envelope genes revealed no additional substitutions. By testing AR3A neutralization sensitivity, we found that H77/JFH1$_{\Delta HVR1/N417D/N532D/Q444R}$ (IC_{50} = 0.000012 μg/ml) was similarly sensitive to AR3A compared with H77/JFH1$_{\Delta HVR1/N417D/N532D}$ (IC_{50} =

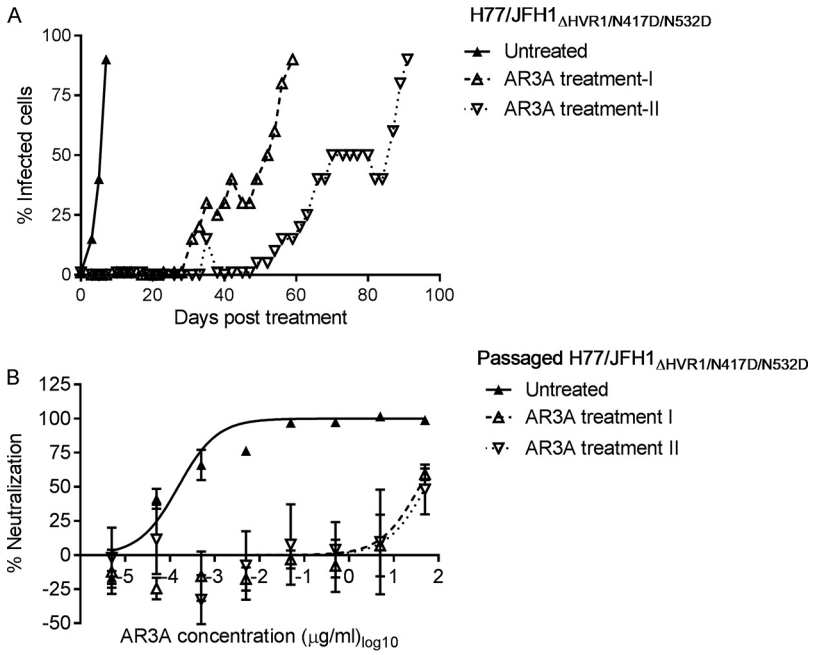

FIG 6 Culturing H77/JFH1$_{\Delta HVR1/N417D/N532D}$ in the presence of NAb AR3A results in high-level resistance. (A) Huh7.5 cells were infected with H77/JFH1$_{\Delta HVR1/N417D/N532D}$ and treated with 20 μg/ml of AR3A antibody during the indicated period of time. Cells from treatments I and II were treated independently and cultured along with the untreated control. The frequencies of infected cells for treatment II were below 0.1% at day 26, and the treatment was interrupted at day 28 until day 35, when the percentage of infected cells increased to 15%. (B) First passages of the virus supernatants taken from treatments I and II and the untreated control were subjected to a dilution series of AR3A from 50 μg/ml to 0.000005 μg/ml, and neutralization was assessed and analyzed as described in the legend of Fig. 1, except that we used three-parameter curve fitting to obtain a sigmoidal dose-response curve.

0.000026 μg/ml). However, H77/JFH1$_{\Delta HVR1/N417D/N532D/L438S/Q444R}$ (IC_{50} $>$ 50 μg/ml) was $>$2,000,000-fold more resistant than H77/JFH1$_{\Delta HVR1/N417D/N532D}$ (Fig. 7B). Thus, we found that L438S increased antibody resistance against AR3A, and the substitution Q444R compensated for impaired virus fitness.

To study if substitutions L438S and Q444R had similar effects on fitness and AR3A resistance for viruses retaining HVR1, we introduced them alone or in combination into H77/JFH1. By transfecting Huh7.5 cells with these recombinants, we observed that H77/JFH1$_{Q444R}$ had titers slightly lower than those of the parental virus. However, as described above for F442Y, L438S abrogated virus infectivity of H77/JFH1 (Fig. 7C).

L438S and F442Y abrogate AR3A binding to E2. To test if the substitutions M345T, L438S, and F442Y impaired AR3A binding to E2 in the context of infected cells, we transfected Huh7.5 cells with H77/JFH1 recombinants without (Fig. 8A) or with (Fig. 8B) HVR1 harboring these substitutions. Transfected cells were fixed and immunostained with E2-specific antibodies AR3A and AP33. The AP33 signal was similar for all viruses, indicating similar E2 stability. While we did not observe decreased AR3A binding for E1 substitution M345T, the antibody signal was at background levels for viruses harboring the E2 substitution L438S or F442Y (Fig. 8). The effect of L438S and F442Y on E2 binding was independent of HVR1, indicating that these substitutions impaired epitope recognition in both HVR1-deleted and parental viruses.

Culturing of J6/JFH1$_{\Delta HVR1}$ in the presence of AR3A results in escape and high-level antibody resistance. As described above for genotype 1a virus H77/JFH1$_{\Delta HVR1}$, we cultured genotype 2a virus J6/JFH1$_{\Delta HVR1}$-infected Huh7.5 cells in the presence of 40 μg/ml of AR3A (treatments I and II) (Fig. 9A and Table 1). To avoid curing the cultures, antibody treatment was interrupted for both cultures at day 27 posttreatment, and supernatants were collected when the infection reached 80% without antibody. Supernatants were used to infect naive Huh7.5 cells, and the cells were

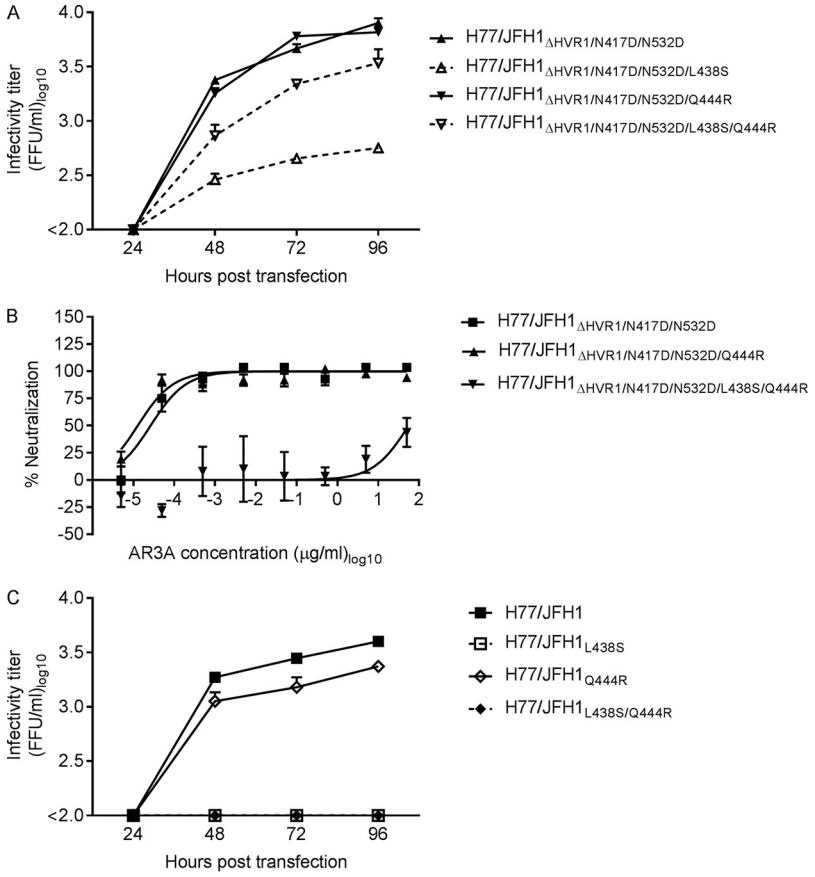

FIG 7 The substitution L438S confers AR3A resistance to H77/JFH1$_{\Delta HVR1/N417D/N532D}$ and abrogates virus infectivity of H77/JFH1 retaining HVR1. (A and C) Huh7.5 cells were transfected with *in vitro*-transcribed RNA of the indicated recombinants without (A) or with (C) HVR1 with single or combined substitutions L438S and Q444R. Supernatants were collected at the indicated time points, and HCV infectivity titers were determined. The lower level of quantification was 100 FFU/ml. (B) First passages of the indicated viruses were subjected to a dilution series of AR3A from 50 μg/ml to 0.000005 μg/ml, and neutralization was assessed and analyzed as described in the legend of Fig. 1, except that we used three-parameter curve fitting to obtain a sigmoidal dose-response curve.

treated with 40 μg/ml of AR3A (Fig. 9B and Table 1). During the second infection, the percentage of infected cells in treatment I reached 80% at day 17 posttreatment (Fig. 9B and Table 1). To avoid curing the treatment II culture, antibody treatment was interrupted at day 75 posttreatment, and the supernatant was collected when the infection reached 80% without antibody (Fig. 9B and Table 1). The supernatant collected during the second infection was used to infect naive Huh7.5 cells in a third infection, and the cells were cultured with 40 μg/ml of AR3A (Fig. 9C and Table 1). This third passage of treatment II reached 80% at day 35 posttreatment (Fig. 9C and Table 1).

Cell supernatants for the control and escaped viruses were used to generate first-passage stocks by infecting naive Huh7.5 cells. By testing AR3A neutralization sensitivity, we found that virus from treatment I (IC_{50} = 18 μg/ml) was ~10,000-fold less sensitive than the untreated virus (IC_{50} = 0.0020 μg/ml) (Fig. 9D and Table 1). Similarly, virus from treatment II (IC_{50} = 3.0 μg/ml) was ~1,500-fold less sensitive than the parental virus (Fig. 9D and Table 1). Sequence analysis of E1/E2 from treatments I and II identified the common substitutions V277A (T1171C) in E1 and D431G (A1633G) in E2 (Table 1). The presence of these envelope substitutions in both cases strongly indicated that one or both were involved in the observed AR3A resistance.

The E2 substitution D431G confers AR3A antibody resistance to J6/JFH1$_{\Delta HVR1}$. The substitutions V277A and D431G were introduced individually or in combination

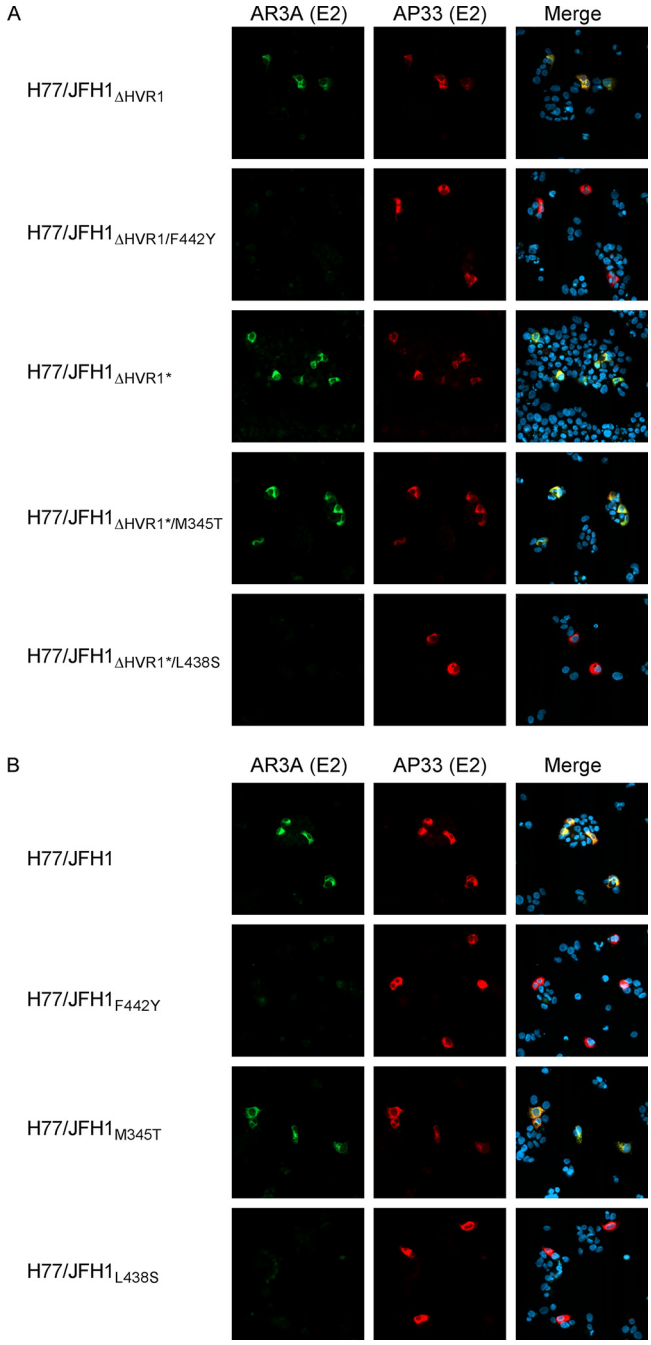

FIG 8 Substitutions F442Y and L438S impair AR3A binding in cells transfected with H77/JFH1 with or without HVR1. Huh7.5 cells were transfected with *in vitro*-transcribed RNA from the indicated recombinants without (A) or with (B) HVR1. Cells were fixed at 48 h posttransfection and stained using the two E2-specific antibodies AR3A and AP33 as described in Materials and Methods. Cell nuclei were visualized with Hoechst 33342. Images were acquired using a Zeiss Axio Observer Z1 microscope. *, H77/JFH1$_{\Delta HVR1/N417D/N532D}$.

into J6/JFH1$_{\Delta HVR1}$. By transfecting Huh7.5 cells with these recombinants, we observed that the substitution V277A did not decrease virus infectivity, whereas D431G did (Fig. 10A). We generated a first-passage stock of all three viruses and sequenced their envelope genes. J6/JFH1$_{\Delta HVR1/V277A/D431G}$ had acquired the additional E1 substitution I347L (A1380C), which has previously been shown to increase the fitness of a related HVR1-deleted virus (36). AR3A neutralization sensitivity was tested, and we found that J6/JFH1$_{\Delta HVR1/V277A}$ (IC_{50} = 0.000057 μg/ml) was similarly as sensitive to AR3A as

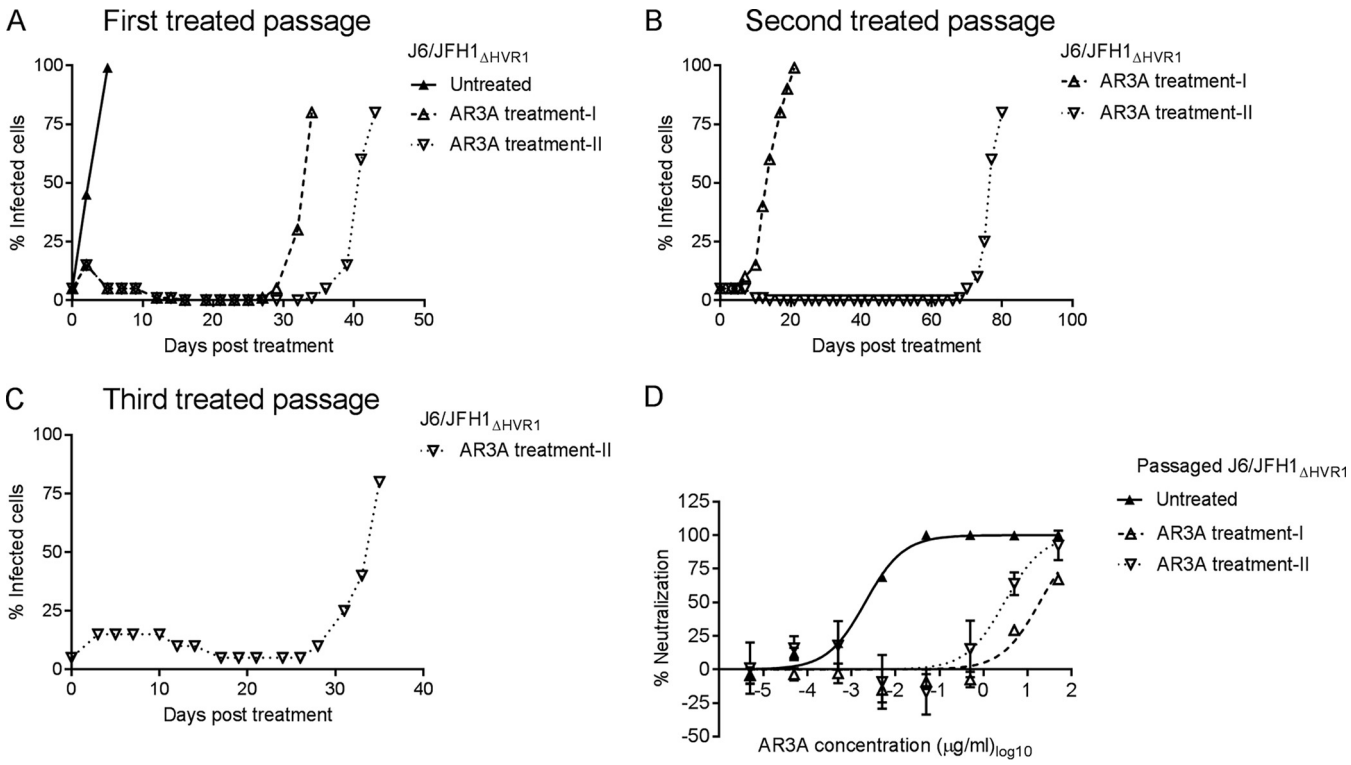

FIG 9 Culturing J6/JFH1$_{\Delta HVR1}$ in the presence of NAb AR3A results in high-level resistance. (A) Huh7.5 cells were infected with J6/JFH1$_{\Delta HVR1}$ and treated with 40 μg/ml of AR3A antibody during the indicated period of time. Cells from treatments I and II were treated independently and cultured along with the untreated control. After day 28, cells from treatments I and II were split without antibody, and the supernatants were collected after the infection had spread to 80% of cells. (B) A second infection was carried out in naive cells. During the second infection, cells from treatment I spread in the presence of 40 μg/ml of AR3A, but cells from treatment II were split without antibody on day 66 postinfection, and the virus supernatant was collected after the infection reached 80% of cells. (C) A third infection was carried out in naive cells. During the third infection, cells from treatment II spread in the presence of 40 μg/ml of AR3A antibody. (D) First passages of the virus supernatant taken from treatments I and II and the untreated control were subjected to a dilution series of AR3A from 50 μg/ml to 0.000005 μg/ml, and neutralization was assessed and analyzed as described in the legend of Fig. 1, except that we used three-parameter curve fitting to obtain a sigmoidal dose-response curve.

J6/JFH1$_{\Delta HVR1}$ (IC_{50} = 0.000071 μg/ml). J6/JFH1$_{\Delta HVR1/D431G}$ (IC_{50} = 1.6 μg/ml) and J6/JFH1$_{\Delta HVR1/V277A/D431G}$ (IC_{50} = 1.9 μg/ml) were ~10,000-fold less sensitive to AR3A neutralization (Fig. 10B). Thus, the E2 substitution D431G conferred AR3A resistance to J6/JFH1$_{\Delta HVR1}$.

Finally, we analyzed the effect of D431G on AR3A epitope binding in the context of infected cells. Huh7.5 cells were transfected with J6/JFH1$_{\Delta HVR1}$ recombinants with or without D431G. At 48 h posttransfection, cells were either fixed and stained with the E2-specific antibodies AR3A and AP33 (Fig. 10C) or prepared for flow cytometry analysis using the same two E2 antibodies (Fig. 10D, with detailed flow cytometric analysis in Fig. 11A to D). Both immunofluorescence microscopy and flow cytometry showed that the AR3A signal was diminished in cells transfected with viral RNA carrying the substitution D431G, indicating that D431G decreased AR3A binding to its epitope. These results were further corroborated by flow cytometry of HEK293T cells transfected with J6 E1/E2 expression plasmids harboring the D431G substitution (Fig. 10D, with detailed flow cytometric analysis in Fig. 11E to H).

D431G does not affect AR3A sensitivity of J6/JFH1 but decreases AR3A binding to E2 in J6/JFH1-infected cells. The substitutions V277A and D431G were introduced into the parental recombinant J6/JFH1. By transfecting Huh7.5 cells, we observed that D431G decreased virus viability, whereas V277A did not affect virus titers (Fig. 12A). Virus supernatants from the peak of infection were used to generate first-passage stocks, and their envelope sequences were confirmed. We found that J6/JFH1 (IC_{50} = 44 μg/ml), J6/JFH1$_{V277A}$ (IC_{50} = 39 μg/ml), and J6/JFH1$_{D431G}$ (IC_{50} = 25 μg/ml) were similarly sensitive to AR3A, whereas J6/JFH1$_{V277A/D431G}$ (IC_{50} = 8.0 μg/ml) was slightly

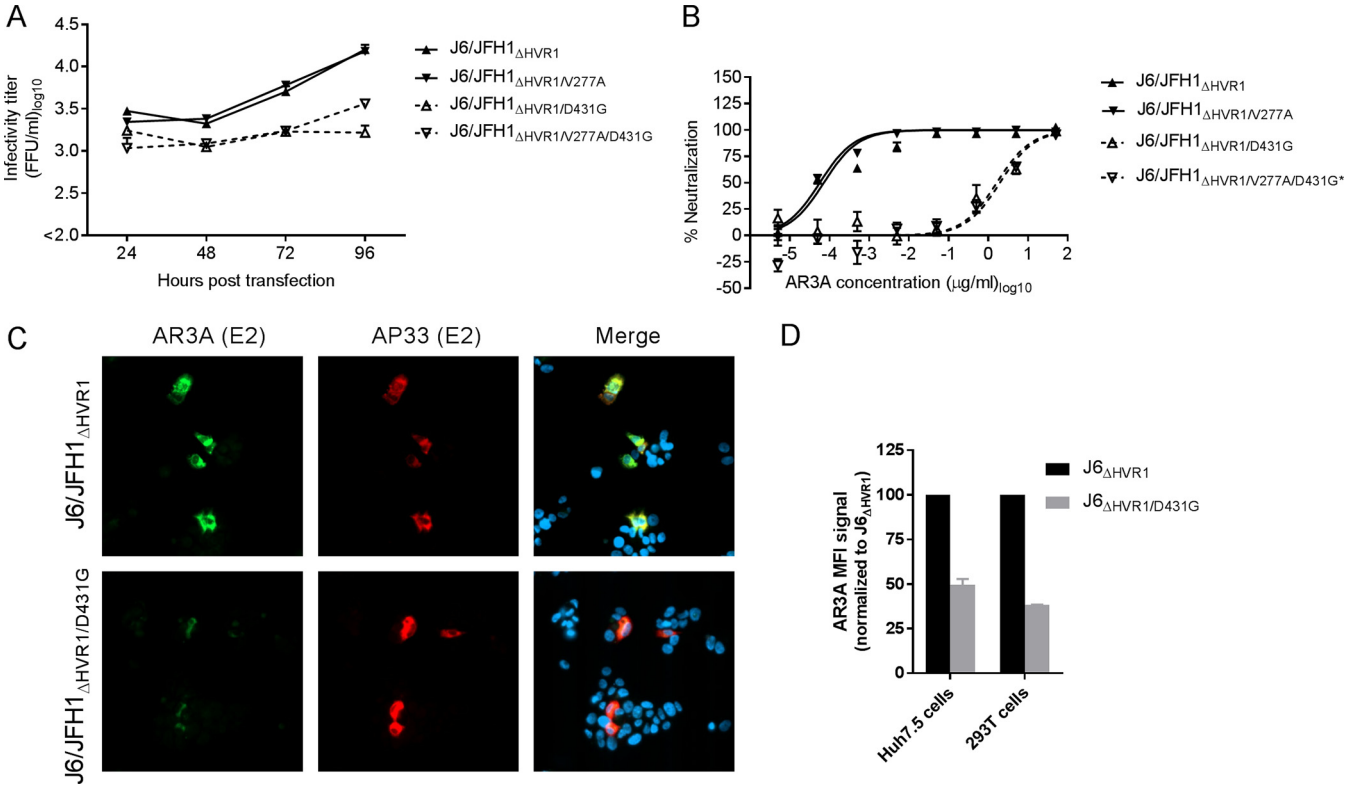

FIG 10 The substitution D431G confers decreased AR3A sensitivity to J6/JFH1$_{\Delta HVR1}$ and decreases AR3A binding to J6 E1/E2$_{\Delta HVR1}$. (A) Huh7.5 cells were transfected with *in vitro*-transcribed RNA of the recombinants with single or combined substitutions V277A and D431G. Supernatants were collected at the indicated time points, and HCV infectivity titers were determined. The lower level of quantification was 100 FFU/ml. (B) First passages of the viruses were subjected to a dilution series of AR3A from 50 μg/ml to 0.000005 μg/ml, and neutralization was assessed and analyzed as described in the legend of Fig. 1, except that we used three-parameter curve fitting to obtain a sigmoidal dose-response curve. *, the indicated virus had the additional E1 substitution I347L. (C) Huh7.5 cells were transfected with *in vitro*-transcribed RNA of the indicated recombinants. Cells were fixed at 48 h posttransfection and stained using the primary antibodies AR3A and AP33 as described in Materials and Methods. Cell nuclei were visualized with Hoechst 33342. Images were acquired using a Zeiss Axio Observer Z1 microscope. (D) Huh7.5 cells were transfected with *in vitro*-transcribed RNA of the indicated core-NS2 J6$_{\Delta HVR1}$ recombinants, and HEK293T cells were transfected with the indicated J6$_{\Delta HVR1}$ E1/E2 expression vectors. Cells were prepared for flow cytometry by releasing them into suspension and fixing and staining them using the primary antibodies AR3A and AP33 as described in Materials and Methods. The AR3A mean fluorescence intensity (MFI) was calculated for the double-positive cell population, as shown in Fig. 11A to D (Huh7.5 cell transfection) and Fig. 11E to H (HEK293T cell transfection), and normalized to the values for J6$_{\Delta HVR1}$. The bars represent the means of data from two independent experiments with standard deviations (SD).

more susceptible to neutralization than the parental virus (Fig. 12B). Thus, while D431G conferred AR3A resistance to HVR1-deleted J6/JFH1, it did not affect AR3A sensitivity of J6/JFH1.

Next, we tested AR3A binding to Huh7.5 cells transfected with J6/JFH1 recombinants with or without D431G. At 48 h posttransfection, cells were either fixed and stained with the E2-specific antibodies AR3A and AP33 (Fig. 12C) or prepared for flow cytometry analysis using the same antibodies (Fig. 12D, with detailed flow cytometric analysis in Fig. 13A to D). Flow cytometry analysis was similarly performed on HEK293T cells transfected with J6 E1/E2 expression plasmids harboring the D431G substitution (Fig. 12D, with detailed flow cytometric analysis in Fig. 13E to H). Surprisingly, both immunofluorescence microscopy and flow cytometry revealed that the AR3A signal was diminished in cells transfected with viral RNA or envelope protein-expressing vectors harboring the substitution D431G, indicating that even in the context of E2 with HVR1, D431G decreased AR3A binding to its epitope.

D431G confers a broad increase in antibody susceptibility of J6/JFH1 retaining HVR1. To investigate if D431G broadly affected antibody susceptibility, we tested neutralization sensitivity against the NAbs AR4A and AR5A (22) of the parental and the HVR1-deleted recombinants with the substitution D431G (Fig. 14). Neutralization susceptibilities of J6/JFH1$_{\Delta HVR1}$ and J6/JFH1$_{\Delta HVR1/D431G}$ were similar for both antibodies. In contrast, J6/JFH1$_{D431G}$ (AR4A IC_{50} = 0.15 μg/ml; AR5A IC_{50} = 0.84 μg/ml) was ~25-fold

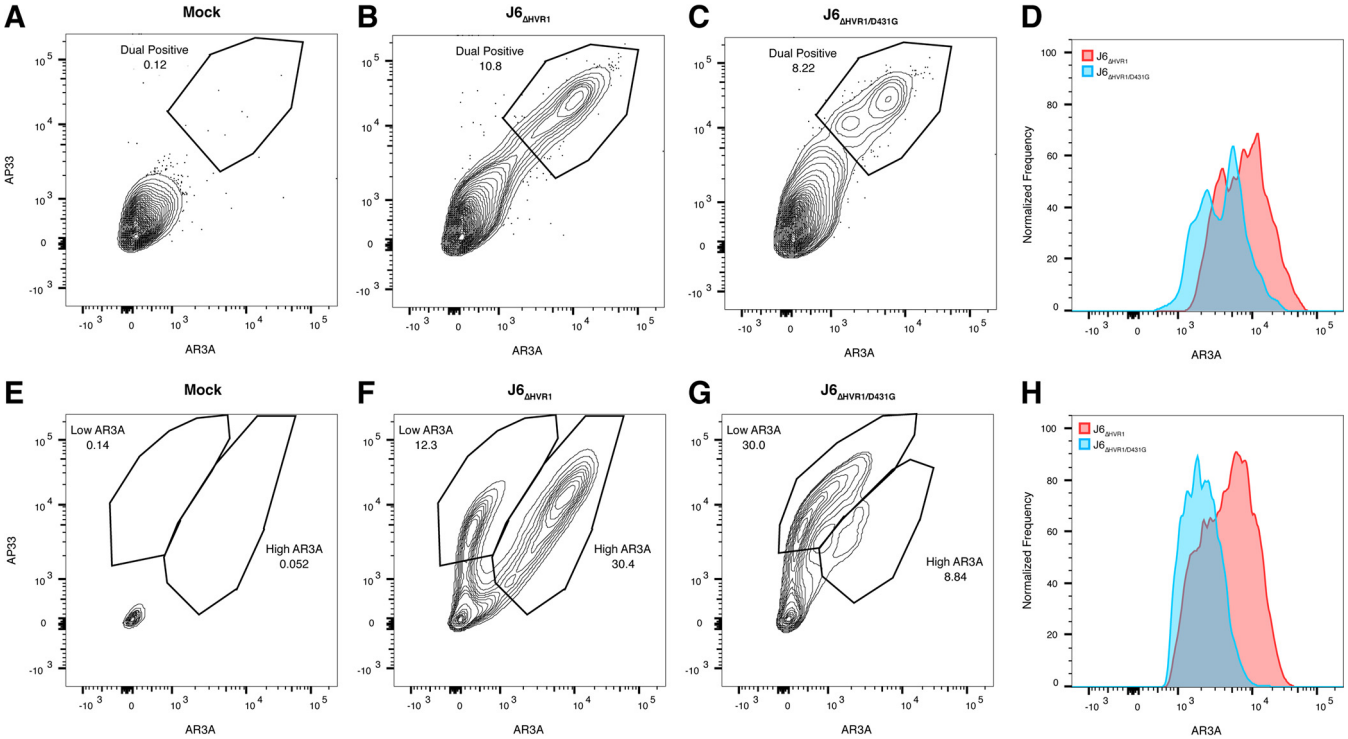

FIG 11 Flow cytometry reveals that D431G decreases AR3A binding to HVR1-deleted E1/E2 in both HCV-infected cells and envelope-expressing cells. (A to C) Flow cytometry analysis of Huh7.5 cells transfected without plasmids (mock) (A) or with recombinant J6/JFH1$_{\Delta HVR1}$ (B) or J6/JFH1$_{\Delta HVR1/D431G}$ (C). Panels show cells double stained with anti-E2 antibody AR3A (secondary stain, Alexa Fluor 488) and the control E2 antibody AP33 (secondary stain, APC). (D) Histograms comparing the normalized fluorescence profiles of envelope variants in the AR3A channel of double-positive cells. (E to G) Flow cytometry analysis of HEK293T cells transfected without a plasmid (mock) (E) or with plasmids expressing HCV E1/E2 variant J6$_{\Delta HVR1}$ (F) or J6$_{\Delta HVR1/D431G}$ (G). Panels show cells double stained with anti-E2 antibody AR3A (secondary stain, Alexa Fluor 488) or the control E2 antibody AP33 (secondary stain, APC). (H) Histograms comparing the normalized fluorescence profiles of envelope variants in the AR3A channel of the double-stained population with higher AR3A binding (High AR3A in the panels). For both flow cytometry experiments, the figure represents data from one of two independent experiments that yielded comparable results.

and ~18-fold more susceptible to AR4A and AR5A, respectively (AR4A IC_{50} = 4.2 μg/ml; AR5A IC_{50} = 15 μg/ml). Thus, D431G conferred a broad increase in neutralization sensitivity to J6/JFH1 but not to the HVR1-deleted counterpart J6/JFH1$_{\Delta HVR1}$.

D431G affects virus breathing without affecting pH and temperature stability of the virus. To investigate how D431G might impact the general neutralization sensitivity of J6/JFH1$_{D431G}$, but not J6/JFH1$_{\Delta HVR1/D431G}$, we tested temperature effects on dynamic properties of envelope epitope availability (e.g., virus breathing), which has previously been shown to impact antibody sensitivity in studies with HCV (40) and related flaviviruses (41, 42). We initially tested the general effect of D431G on envelope breathing by performing neutralization experiments at 4°C, 37°C, and 40°C using the E2 antibody H77.39 (previously used to assess temperature-dependent neutralization of HCV [40]). For H77.39, the level of neutralization of J6/JFH1 was ~3-fold higher at 40°C (IC_{50} = 9.2 μg/ml) than at 37°C (IC_{50} = 30 μg/ml) (Fig. 15A). In contrast, the level of neutralization of J6/JFH1$_{D431G}$ was ~27-fold higher at 40°C (IC_{50} = 0.49 μg/ml) than at 37°C (IC_{50} = 13 μg/ml) (Fig. 15B), suggesting that D431G aided in destabilizing closed, neutralization-resistant, envelope protein conformations. Interestingly, virtually no effect on neutralization between 37°C and 40°C was observed for J6/JFH1$_{\Delta HVR1}$ and J6/JFH1$_{\Delta HVR1/D431G}$ (Fig. 15C and D), indicating that the level of virus breathing was lower for HVR1-deleted viruses and, perhaps consequently, unaffected by D431G.

By performing similar breathing experiments at 4°C, 37°C, and 40°C with AR3A, we observed that the level of neutralization of J6/JFH1$_{D431G}$ at 37°C was ~4-fold higher (IC_{50} = 44 μg/ml) than for J6/JFH1 (IC_{50} = 170 μg/ml) (Fig. 15E and F). However, in contrast to H77.39 neutralization, we observed a 5-fold decrease in AR3A sensitivity of J6/JFH1$_{D431G}$ at 40°C (IC_{50} = 3.1 μg/ml) compared to J6/JFH1 (IC_{50}= = 0.62 μg/ml)

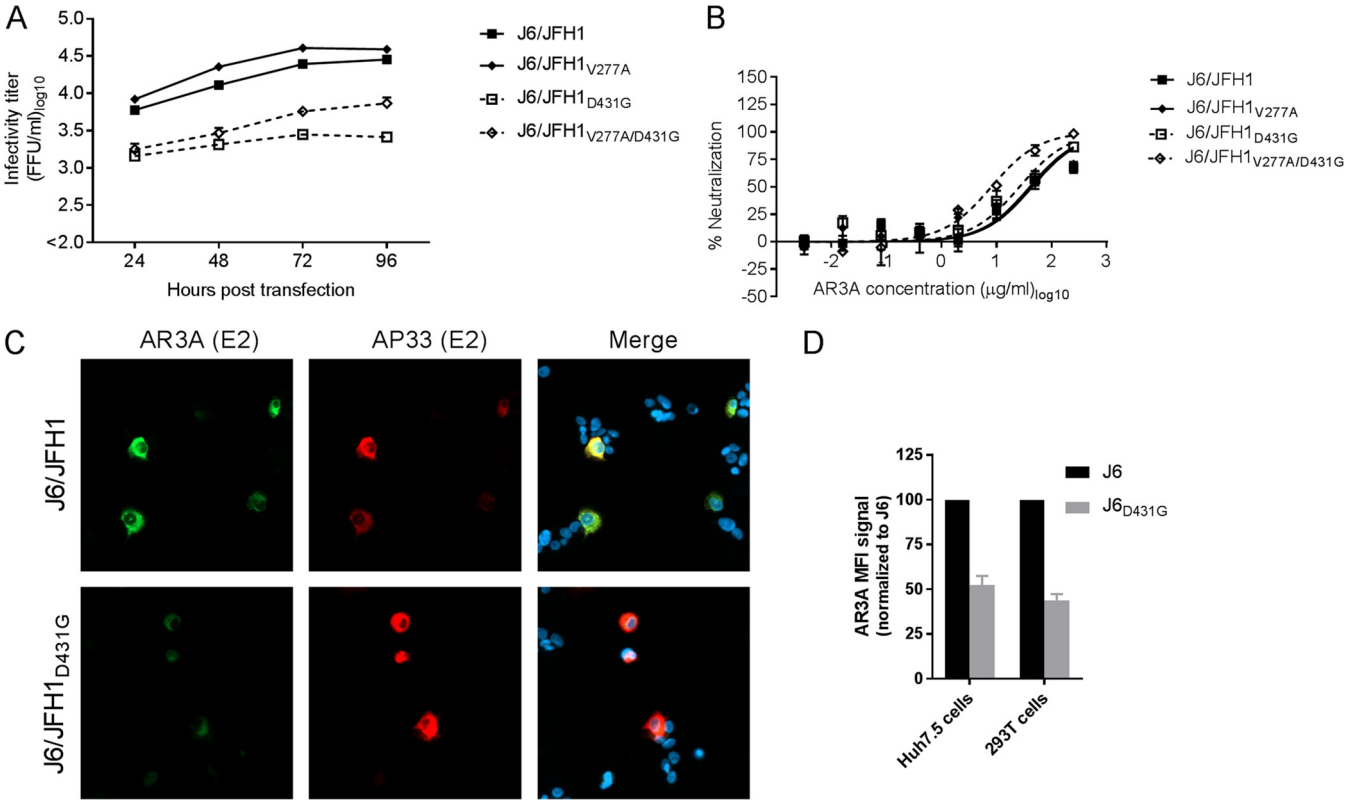

FIG 12 The substitution D431G does not affect AR3A sensitivity of J6/JFH1 but decreases AR3A binding to J6 E1/E2. (A) Huh7.5 cells were transfected with *in vitro*-transcribed RNA of the indicated recombinants with single or combined mutations. Supernatants were collected at the indicated time points, and HCV infectivity titers were determined. The lower level of quantification was 100 FFU/ml. (B) First passages of the viruses with single or combined mutations were subjected to a dilution series of AR3A from 250 μg/ml to 0.0032 μg/ml, and neutralization was assessed and analyzed as described in the legend of Fig. 1, except that we used three-parameter curve fitting to obtain a sigmoidal dose-response curve. (C) Huh7.5 cells were transfected with *in vitro*-transcribed RNA of the indicated recombinants. Cells were stained using AR3A and AP33 as described in the legend of Fig. 10. (D) Huh7.5 cells were transfected with *in vitro*-transcribed RNA of the indicated core-NS2 J6 recombinants, and HEK293T cells were transfected with the indicated J6 E1/E2 expression vectors. Cells were prepared for flow cytometry by releasing them into suspension and fixing and staining them using the primary antibodies AR3A and AP33 as described in Materials and Methods. The AR3A mean fluorescence intensity (MFI) was calculated for the double-positive cell population, as shown in Fig. 13A to D (Huh7.5 cell transfection) and Fig. 13E to H (HEK293T cell transfection), and normalized to the values for J6. The bars represent the means of data from two independent experiments with SD.

(Fig. 15E and F), which better reflects our finding that the AR3A epitope is partially compromised by D431G. AR3A neutralization of J6/JFH1$_{\Delta HVR1}$ and J6/JFH1$_{\Delta HVR1/D431G}$ was virtually unaffected by temperature, but we confirmed that J6/JFH1$_{\Delta HVR1/D431G}$ was less sensitive to AR3A neutralization (Fig. 15G and H).

Finally, we tested whether D431G affected the temperature and pH stability of the virions. Virus infectivity was similarly affected for both viruses at all three temperatures (Fig. 16A and B) and at all three pHs (Fig. 16C).

Substitution D431G, but not M345T, decreases SR-BI dependency. To test whether viable AR3A resistance substitutions conferred differences in receptor usage, we incubated Huh7.5 cells with serially diluted blocking antibodies against HCV coreceptors CD81 and SR-BI (38) prior to infecting the cells with H77/JFH1$_{M345T}$ or J6/JFH1$_{D431G}$ and the respective control viruses. The viruses had similar CD81 dependency (Fig. 17A to D). However, in the presence of HVR1, we found that D431G decreased HCV dependency on SR-BI, whereas M345T did not (Fig. 17E to H).

Common polymorphisms at positions 431 and 442 have isolate-dependent effects on virus fitness and do not confer high-level AR3A resistance. Given the relatively high frequency of the polymorphisms F442I and F442L in HCV isolates, we engineered H77, J6, JFH1 (genotype 2a), and T9 (genotype 2a) HCV recombinants with these substitutions. In addition, others have reported modulation of NAb resistance of H77 or JFH1 harboring substitutions at position 431 (26, 29). Therefore, we also

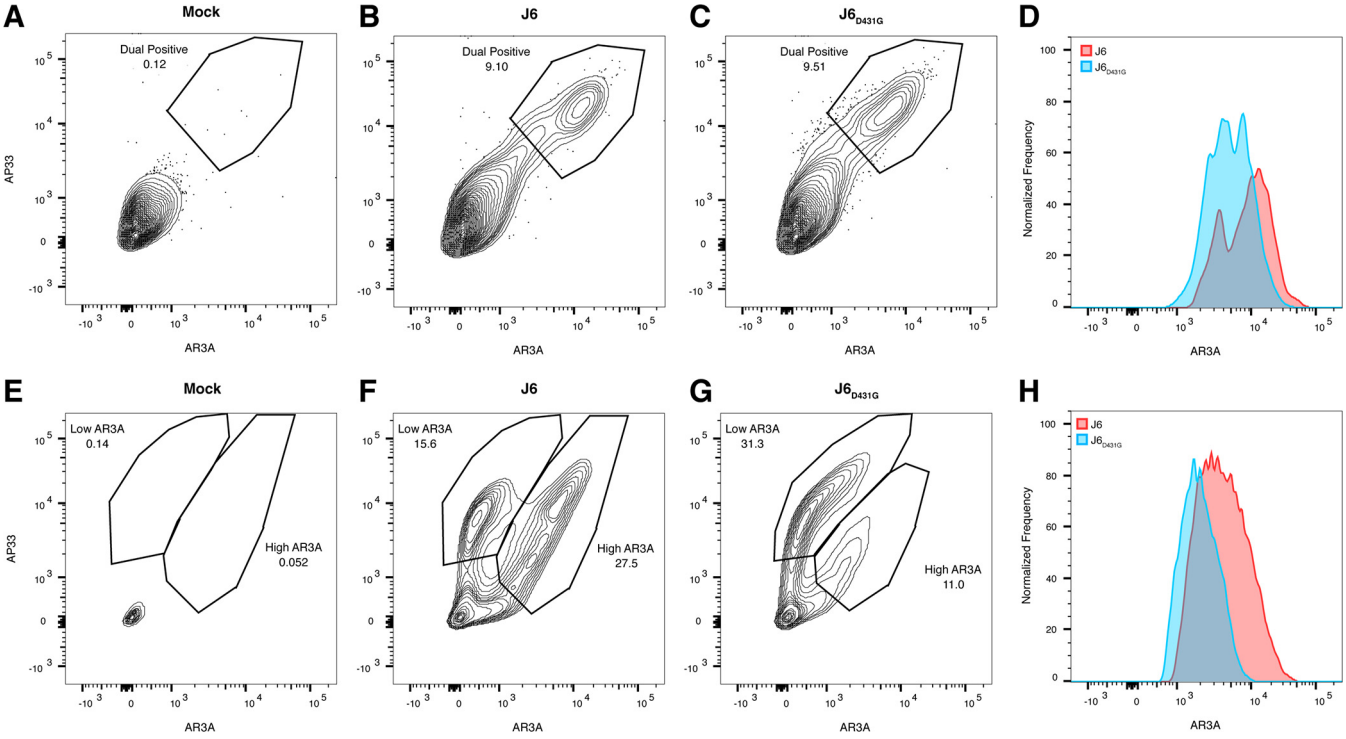

FIG 13 Flow cytometry reveals that D431G decreases AR3A binding to full-length E1/E2 in both HCV-infected cells and envelope-expressing cells. (A to C) Flow cytometry analysis of Huh7.5 cells transfected without plasmids (mock) (A) or with recombinant J6/JFH1 (B) or J6/JFH1$_{D431G}$ (C). Panels show cells double stained with anti-E2 antibody AR3A (secondary stain, Alexa Fluor 488) and the control E2 antibody AP33 (secondary stain, APC). (D) Histograms comparing the normalized fluorescence profiles of envelope variants in the AR3A channel of double-positive cells. (E to G) Flow cytometry analysis of HEK293T cells transfected without a plasmid (mock) (E) or with plasmids expressing HCV E1/E2 variant J6 (F) or J6$_{D431G}$ (G). Panels show cells double stained with anti-E2 antibody AR3A (secondary stain, Alexa Fluor 488) or the control E2 antibody AP33 (secondary stain, APC). (H) Histograms comparing the normalized fluorescence profiles of envelope variants in the AR3A channel of the double-stained population with higher AR3A binding (High AR3A in the panels). For both flow cytometry experiments, the figure represents data from one of two independent experiments that yielded comparable results.

introduced substitutions at position 431 in these same four HCV recombinants. By transfecting Huh7.5 cells with these recombinants, we observed greatly decreased fitness of all modified J6/JFH1 recombinants, moderate fitness loss of H77/JFH1 and JFH1 recombinants (except JFH1$_{D431G}$), and minimal fitness loss of T9/JFH1 recombinants (Fig. 18A to D).

First passages in Huh7.5 cells of H77/JFH1 and J6/JFH1 harboring position 431 and 442 variants did not spread, indicating impaired fitness. However, unmodified viruses and position 431 and 442 variants of JFH1 and T9/JFH1 spread in 8 to 13 days postinfection, except for JFH1$_{D431G}$ and JFH1$_{F442I}$, which spread in 27 and 18 days

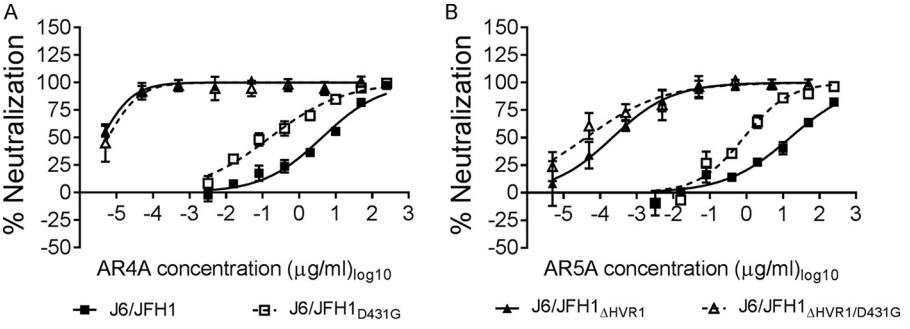

FIG 14 D431G increases antibody susceptibility against the NAbs AR4A and AR5A in J6/JFH1 retaining HVR1. First passages of the indicated viruses were subjected to a dilution series of antibodies AR4A (A) and AR5A (B) from 50 μg/ml to 0.000005 μg/ml or from 250 μg/ml to 0.0032 μg/ml, and neutralization was assessed and analyzed as described in the legend of Fig. 1.

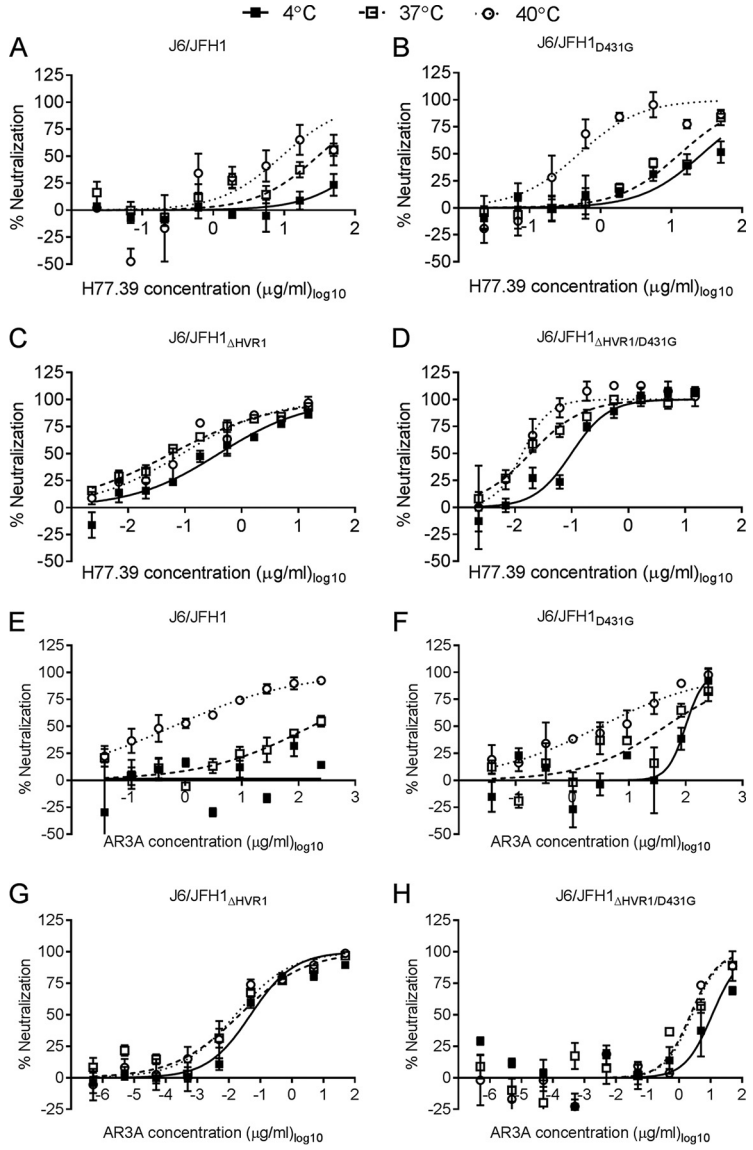

FIG 15 D431G alters temperature modulation of antibody neutralization of J6/JFH1 but not J6/JFH1$_{\Delta HVR1}$. First passages of the virus J6/JFH1 (A and E), J6/JFH1$_{D431G}$ (B and F), J6/JFH1$_{\Delta HVR1}$ (C and G), or J6/JFH1$_{\Delta HVR1/D431G}$ (D and H) were subjected to a dilution series of the antibody H77.39 (A to D) or AR3A (E to H). The virus-antibody mixes along with virus only were incubated for 1 h at 4°C, 37°C, and 40°C. The virus-antibody mixes were chilled to 4°C and added to prechilled Huh7.5 cells, and cells were infected by spinoculation during 2 h at 500 RCF. Forty-eight hours postinfection, the cells were immunostained, and the number of FFU per well was counted. Error bars represent standard errors of the means of data from three replicates normalized to data from six replicates of virus only. When an effect was observed, the data were analyzed using four-parameter curve fitting to obtain a sigmoidal dose-response curve.

postinfection, respectively. Sequencing of the envelope revealed that all viruses had retained the specific substitutions and that most viruses had not acquired additional envelope substitutions, except JFH1$_{D431G}$ and JFH1$_{F442I}$, which had acquired the substitutions T329A (A1326G) in E1 and L640I/l (C2259A/c) in E2, respectively. Next, we tested neutralization sensitivity against AR3A (Fig. 19A and B). JFH1 and T9/JFH1 F442I/L variants had 20- to 59-fold increased AR3A sensitivities. D431E conferred marginal AR3A resistance (2.3- to 2.9-fold) to both viruses, and D431G conferred 4.2-fold increased sensitivity to JFH1, whereas it conferred 7.1-fold increased resistance to T9/JFH1. By testing neutralization sensitivity against AR4A, we observed that F442

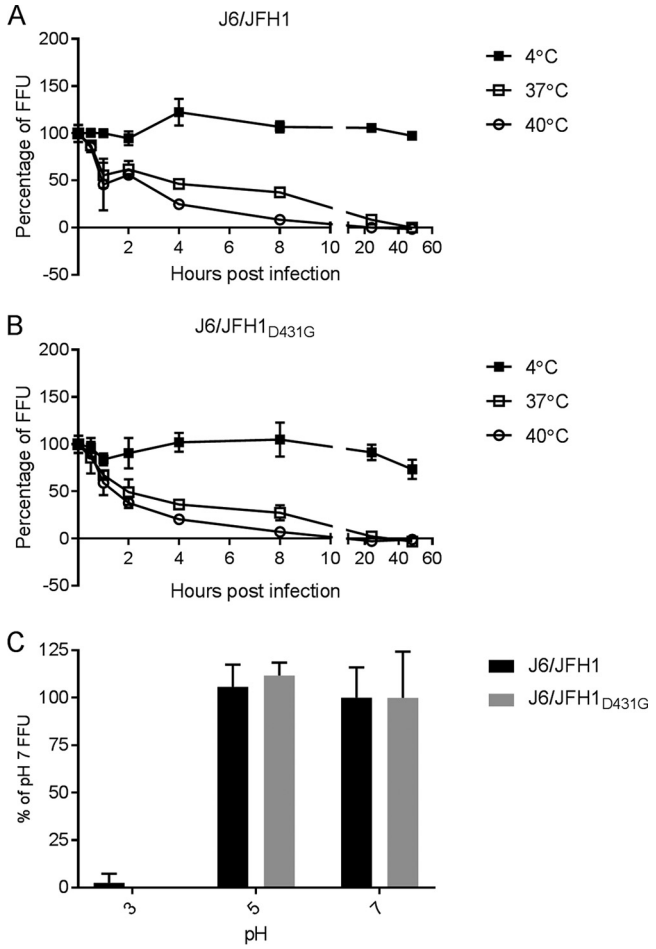

FIG 16 D431G does not affect J6/JFH1 stability to temperature and acidic pH. (A and B) First passages of the viruses J6/JFH1 (A) and J6/JFH1$_{D431G}$ (B) were incubated at 4°C, 37°C, and 40°C for 0.5, 1, 2, 4, 8, 24, and 48 h, and the temperature was adjusted to 37°C prior to infection. Viruses were added to Huh7.5 cells; and after a 48-h infection, the cells were immunostained, and the number of HCV FFU per well was counted. FFU values are the means of data from three replicates. (C) First passages of the indicated viruses were incubated for 15 min at pH 3, 5, or 7. The pH was raised to 7, viruses were added to Huh7.5 cells, and after a 48-h infection, the cells were immunostained and the number of FFU per well was counted. FFU values are the means of data from three replicates, and they are represented as percentages of the mean FFU at pH 7. Error bars represent standard errors of the means.

substitutions broadly increased neutralization sensitivity, whereas only D431G had this effect and only for JFH1 (Fig. 19C and D).

DISCUSSION

Despite the improvements made in HCV therapy by the advent of DAAs, a prophylactic vaccine remains fundamental to HCV eradication. The high variability among HCV genotypes and the ability of the virus to evade the immune system have so far impeded the development of a pangenotypically protective vaccine (5, 43–45). The NAb AR3A showed broad neutralization activity across HCV genotypes (14, 21, 23, 46). However, little is known about the AR3A barrier to resistance, which is the subject of this study.

First, we analyzed alanine substitutions that we previously showed to decrease AR3A binding to H77 E1/E2 (21). This was confirmed in a recent study showing that they primarily affected all AR3-specific NAbs, with the exception of N540A, which apparently caused global folding problems at the recombinant protein level (47). Only a few substitutions, namely, S424A, P525A, and N540A, were viable in H77/JFH1 cell culture and displayed low-level resistance against AR3A. The high fitness of H77/JFH1$_{N540A}$ was particularly surprising due to the above-mentioned effect of this substitution on global

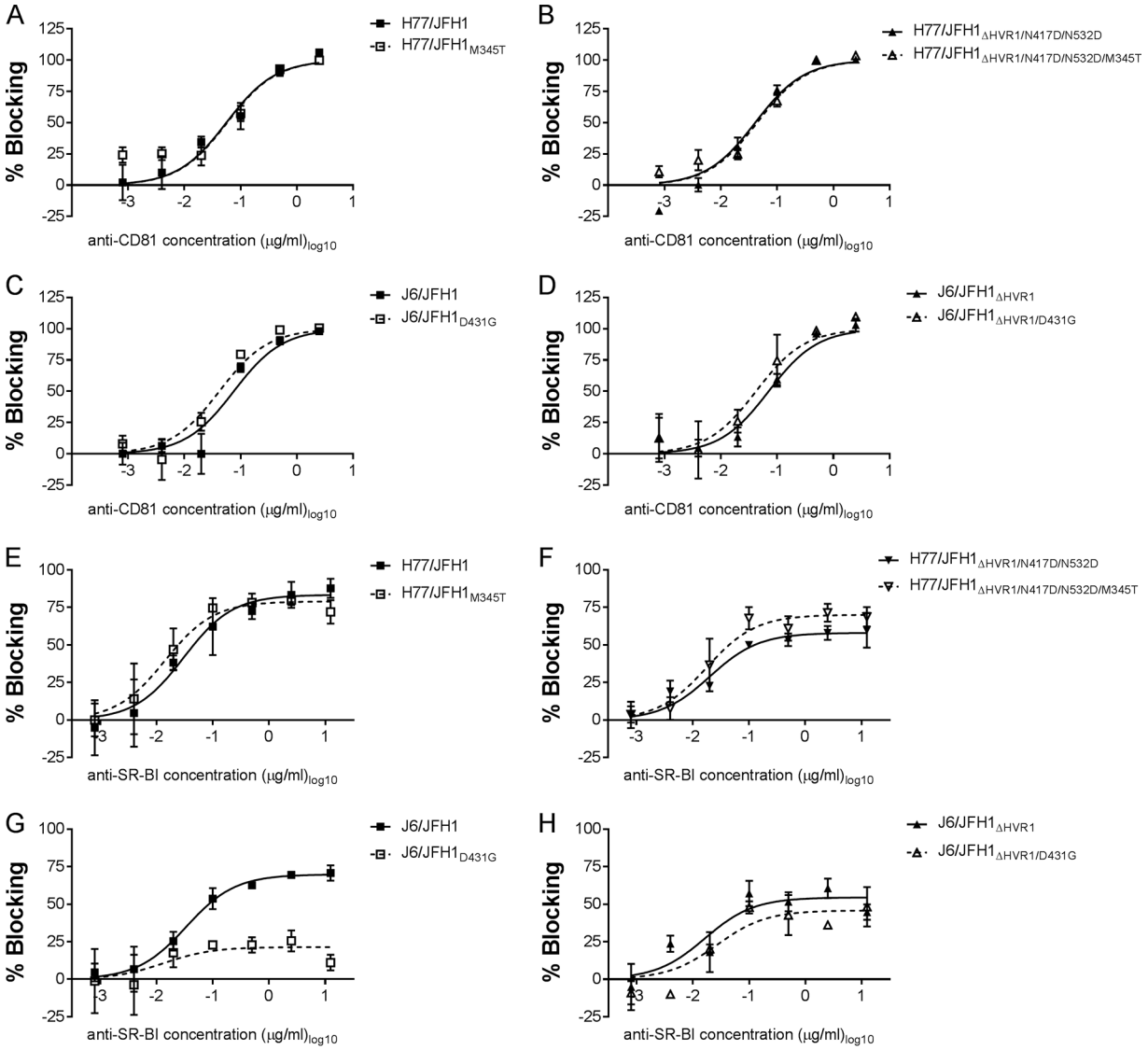

FIG 17 The substitutions M345T and D431G do not alter CD81 dependency, whereas D431G reduces SR-BI dependency in the presence of HVR1. Huh7.5 cells were incubated with dilution series of antibodies that specifically block the interaction between HCV and CD81 (A to D) or SR-BI (E to H) in quadruplicates with 8 wells of virus only prior to addition of the indicated HCV recombinants. The data were analyzed using three-parameter dose-response curves (GraphPad Prism 7.02). Error bars represent SEM.

recombinant E1/E2 protein folding (47). It is possible that other recently reported alanine substitutions that affect AR3A binding, such as W616A, Q673A, and S711A, could have produced viable resistant viruses (47).

We identified the substitution M345T by culturing H77/JFH1 in the presence of AR3A. M345T increased virus fitness and AR3A resistance but only in the presence of HVR1. Threonine (T) at this position is extremely rare, but this position displays a great deal of variation both within genotype 1 and across genotypes (data from the Los Alamos HCV sequence database) (Table 1). Previously, we have observed that high-fitness viruses could elude antibody neutralization without developing resistance substitutions (24, 31), either by rendering the antibody unable to neutralize enough of the virus particles to prevent spread in culture or by high levels of cell-to-cell spread (32, 33). It is therefore possible that the virus benefited from such an effect to spread in the presence of AR3A. However, this is the first reported case of the emergence of an AR3A-specific resistance substitution *in vitro*. It is of note that M345T is within the E1 protein. This is intriguing, as AR3A recognizes E2 when expressed without E1. Our

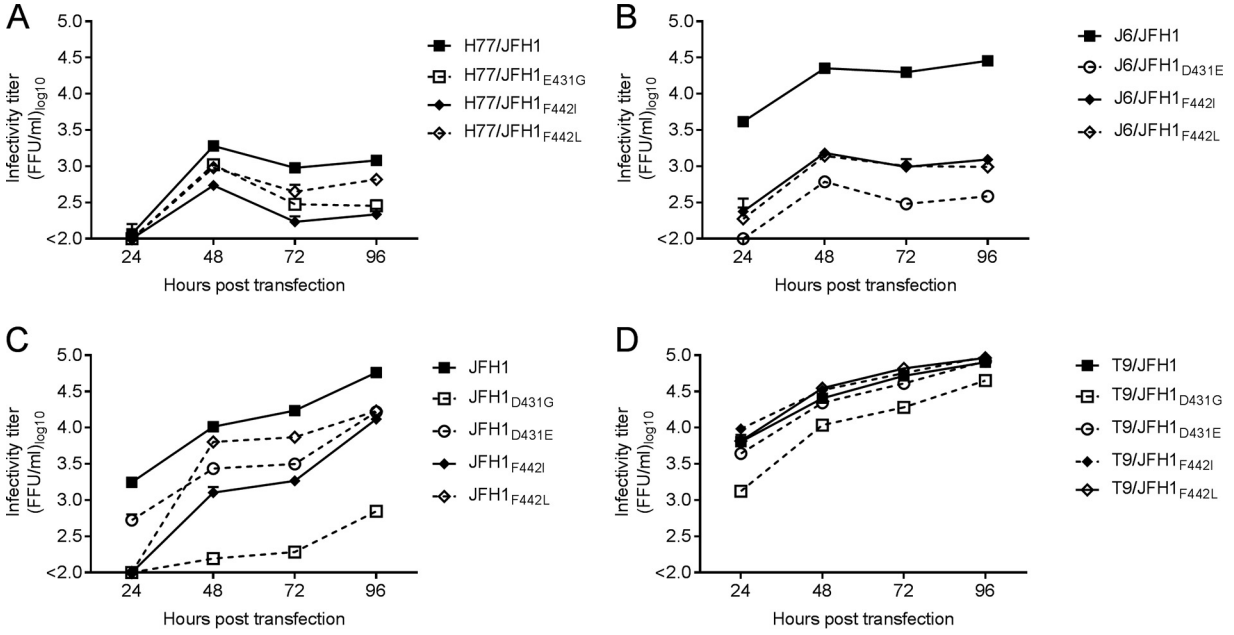

FIG 18 Polymorphisms at positions 431 and 442 have isolate-dependent effects on HCV viability. Huh7.5 cells were transfected with *in vitro*-transcribed RNA of H77/JFH1 (A), J6/JFH1 (B), JFH1 (C), or T9/JFH1 (D) harboring the indicated substitutions at position 431 or 442. Supernatants were collected at the indicated time points, and HCV infectivity titers were determined. The lower level of quantification was 100 FFU/ml.

findings suggest that the interaction between E1 and E2 could be modulating AR3A epitope availability in an HVR1-dependent manner. This is further supported by recent findings that alanine substitutions in E1, such as V277A, I311A, and I313A, decrease AR3A binding to E1/E2 by ~50% (47). However, the current lack of reliable models for the E1/E2 protein complex makes it unfeasible to verify this hypothesis.

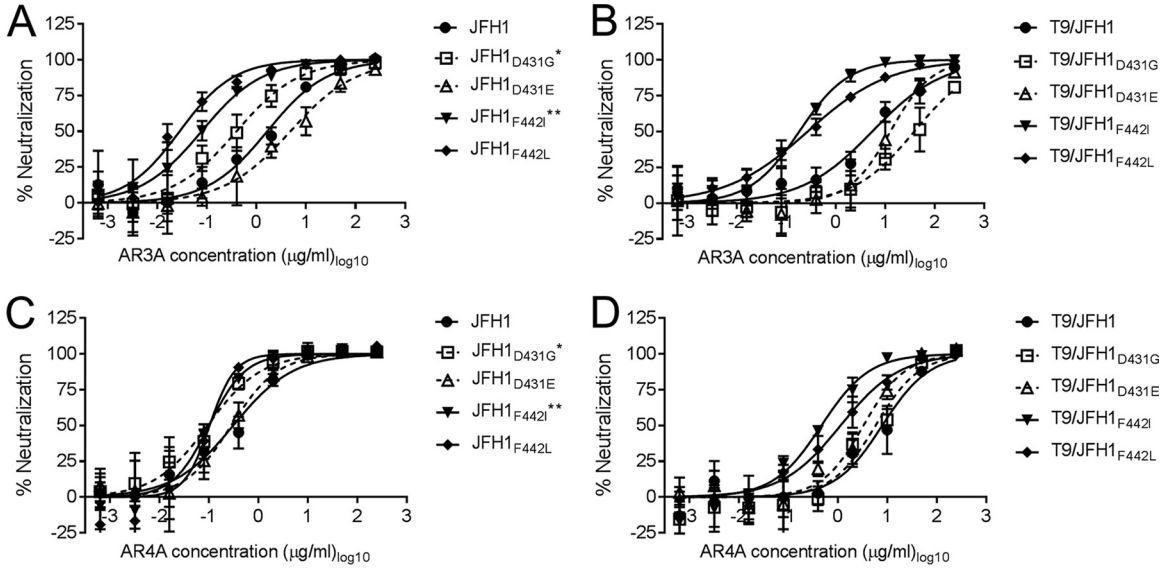

FIG 19 Polymorphisms at positions 431 and 442 broadly affect HCV neutralization sensitivity without conferring high-level AR3A resistance. First passages of the indicated JFH1 or T9/JFH1 recombinants were subjected to a dilution series of antibody AR3A (A and B) or AR4A (C and D). The virus-antibody mixes along with virus only were added to Huh7.5 cells for 4 h prior to washing and the addition of fresh medium. Following a total of 48 h of infection, the cells were immunostained, and the number of FFU per well was counted as described in Materials and Methods. Error bars represent standard errors of the means of data from four replicates normalized to data from 8 replicates of virus only. The data were analyzed using four-parameter curve fitting to obtain a sigmoidal dose-response curve. *, the virus had the additional E1 substitution T329A; **, the virus had the additional E2 substitution L640I.

We identified the substitutions L438S and F442Y by culturing H77/JFH1$_{\Delta HVR1}$ in the presence of AR3A. These substitutions are in the AR3 epitope formed by the regions spanning positions 396 to 424, 436 to 447, and 523 to 540, in which G523, G530, and G535 serve functions in CD81 binding (21, 48). Positions 438 and 442 display a high degree of variation, but neither substitution is observed in the Los Alamos HCV sequence databases (Table 1). Interestingly, we recently reported that alanine substitutions at these positions reduced AR3A binding to H77 E1/E2 and were critical for interactions with CD81 (47). L438S and F442Y greatly increased AR3A resistance for H77/JFH1$_{\Delta HVR1}$. In addition, although Q444R is also within the AR3A binding regions, it compensated for the L438S fitness decrease without affecting AR3A susceptibility. Polymorphisms at position L438 have been associated with conferring broad neutralization resistance, including the AR3A-related antibody AR3C (49). Interestingly, L438S and F442Y were tolerated when HVR1 was deleted, but both substitutions completely abrogated virus infectivity for the parental virus H77/JFH1. Positions L438 and F442 have been implicated in viral entry and direct interaction of E1/E2 with the cellular receptor CD81 (47, 50), suggesting that the loss in viral fitness observed for H77/JFH1 could be due to CD81-specific entry deficiencies. This, in turn, implies differences in CD81 interactions for viruses with and without HVR1. Interestingly, upon engineering H77/JFH1, J6/JFH1, JFH1, and T9/JFH1 HCV recombinants with the common polymorphisms F442I and F442L, we observed dramatically decreased virus viability of H77/JFH1 and J6/JFH1. For JFH1 and T9/JFH1, both substitutions increased virus neutralization susceptibility against both AR3A and AR4A, indicating that these polymorphisms did not confer AR3A resistance and indeed suggesting that they broadly sensitized these viruses to neutralization.

We identified the substitution D431G by culturing J6/JFH1$_{\Delta HVR1}$ in the presence of AR3A. Some variation is observed at this position across genotypes, but it is extremely conserved within genotype 2, as only 3% have glutamic acid (E) instead of aspartic acid (D), which are highly similar amino acids (data from the Los Alamos HCV sequence database) (Table 1). D431G increased AR3A-specific resistance for J6/JFH1$_{\Delta HVR1}$, but did not alter AR3A sensitivity of J6/JFH1, when assayed at 37°C. This may be due to a secondary effect of D431G, which, in the context of J6/JFH1 but not J6/JFH1$_{\Delta HVR1}$, confers a broad neutralization sensitivity increase, which could negate the D431G effect on the AR3A epitope. By immunostaining, we observed decreased binding to cells infected with J6/JFH1 with and without HVR1 harboring D431G. The implication of position 431 in the AR3A epitope was not found for H77 in our recent alanine scanning study of AR3A binding to H77 with E431A (H77 has E431, whereas J6 has D431) (47). However, we reported decreased binding for the proximal substitutions N428A and C429A (47), supporting that position 431 is an important part of the AR3A epitope and validating the relevance of our approach to studying antibody resistance. Substitutions at position 431 have also been shown to alter sensitivity to multiple antibodies, such as CBH-5, CBH-7, and, most notably, CBH-2 (29). Upon engineering H77/JFH1, J6/JFH1, JFH1, and T9/JFH1 HCV recombinants with substitutions to G or E (when relevant) at position 431, we observed dramatically decreased virus viability of H77/JFH1 and J6/JFH1. In JFH1, D431G did not increase AR3A resistance and appeared to broadly increase neutralization sensitivity, as observed for J6/JFH1. In T9/JFH1, D431G conferred low-level AR3A-specific resistance (7.1-fold), suggesting that D431G is potentially a viable escape substitution in T9. Interestingly, another substitution close to the AR3 epitope, G451R, has been shown to increase general susceptibility to neutralization (51), and L438V has been shown to increase neutralization susceptibility against AR4A and HC33.4 (52). Taken together, these results suggest that several substitutions in this region of E2 broadly influence neutralization sensitivity of HCV. Our data also indicate that this modulation depends on the presence of HVR1.

As stated above, the AR3A resistance substitutions that we observed are nonexistent in the Los Alamos HCV sequence database, suggesting that they are rare in natural HCV infection. This is perhaps because of their effects on virus fitness and NAb sensitivity. It is interesting that natural polymorphisms at positions 431, 438, and 442 have recently

been implicated in broadly influencing neutralization sensitivity of HCV genotype 1 (49, 52), which we verify here in the case of D431G (for J6/JFH1 and JFH1) and F442I/L (for JFH1 and T9/JFH1). This further strengthens the conclusion that alteration of the AR3A epitope frequently leads to broad increases in HCV neutralization sensitivity. To investigate the mechanism of the broad increase in neutralization sensitivity conferred by D431G, we tested the effect of the substitution on virus breathing. Virus breathing increases binding of antibodies to less-accessible epitopes by perturbation of a compact protein structure at higher temperatures (41, 42, 53). HCV antibody susceptibility has been shown to increase with increasing time of incubation and temperature, indicating that the E1/E2 glycoproteins are subject to breathing (40). Our data support that both higher temperatures and the substitution D431G shift the equilibrium toward open E1/E2 conformations and, consequently, to a more neutralization-sensitive phenotype. Thus, the substitution D431G potentially has two effects on J6/JFH1. One is the direct reduction in AR3A binding by changing of the epitope. The other is an indirect and broad effect (e.g., presumably including the AR3A epitope) of increasing HCV neutralization sensitivity by shifting the equilibrium toward more-open E1/E2 conformations. While the effect at the epitope level remains constant across temperatures, the indirect effect on neutralization sensitivity becomes less and less important as temperature increases to the point where most of the E1/E2 adopts an open conformation. Consequently, the AR3A epitope-specific effect becomes more important than the cross-epitope effect on breathing. This fits our data, as we observe increased AR3A sensitivity of J6/JFH1$_{D431G}$ at 4°C and at 37°C compared with J6/JFH1, whereas the opposite is true at 40°C. The fact that D431G did not have this effect in the absence of HVR1, for which neutralization sensitivity is high irrespective of temperature, indicates that HVR1 is involved in virus breathing. This is particularly interesting as it may suggest a novel mechanism of HVR1-mediated neutralization protection involving an HVR1-dependent effect on stabilizing more-closed conformations in the E1/E2 breathing model, which is different from HVR1 physically covering the protected epitopes.

We also found that neither M345T nor D431G altered CD81 dependency of HCV, which is interesting given our recent report that E431A decreased CD81 interactions at the protein level (47). However, it should be noted that viruses with D431G had decreased fitness, which could be indicative of a decreased capacity to interact correctly with CD81.

M345T did not affect SR-BI dependency, whereas D431G did but only in the presence of HVR1. As D431G also broadly modulated NAb sensitivity, this suggests a link between SR-BI and neutralization sensitivity, as previously reported (54, 55). Furthermore, it seems that D431G shares properties with previously described envelope substitutions that simultaneously alter neutralization sensitivity and SR-BI dependency of HCV (51, 52, 56). Together with our finding that modulation of broad neutralization dependency is HVR1 dependent, this indicates a role of HVR1 in this novel mechanism of antibody evasion. The fact that D431G also increased virus breathing indicates that SR-BI dependency and virus breathing may be linked.

We previously reported that substitutions at position L665 were well tolerated and greatly increased AR5A-specific resistance for viruses of the 6 major HCV genotypes (24). Here, we find that the resistance substitutions M345T, D431G, L438S, and F442Y have an HVR1-dependent effect on virus resistance and fitness. Interestingly, D431G as well as the polymorphisms F442I and F442L increased broad NAb susceptibility, as recently reported by us for AR4A antibody escape substitution I696N (57) and by others for escape substitutions in epitope I (58). Our data suggest that AR3A has a higher barrier to resistance than AR5A, due to deleterious effects on virus fitness and/or increased sensitivity to other NAbs.

Rational vaccine design, in which select epitopes are emphasized, has so far had limited success for HCV. One approach relies on the induction of NAbs against extended or cyclic versions of linear epitopes (59–61). These approaches cannot readily be adapted to AR3A, as it has a conformational, nonlinear epitope. A more promising approach would be to modify existing vaccine candidates, such as E1/E2 or soluble E2,

to increase the exposition of AR3A while potentially decreasing the availability of other epitopes that either are poorly conserved or have low barriers to resistance. Such approaches have already provided some success for HCV (62–64) and for HIV vaccine candidates (65), but these approaches would benefit greatly from a better understanding of the E1/E2 protein complex.

In conclusion, by culturing viruses in the presence of the clinically relevant NAb AR3A, we identified several escape substitutions. Of these, M345T was a classic *de novo* escape substitution, as it conferred low-level antibody-specific resistance in viruses retaining HVR1. However, we identified several substitutions which affected epitope-specific binding but were either nonviable or nonresistant (at 37°C) in the presence of HVR1. Our data suggest that substitutions that broadly regulate NAb sensitivity of HCV and modulate SR-BI dependency of the virus do so in an HVR1-dependent manner. Our data also suggest that virus breathing is involved in these processes and consequently also in HVR1-mediated NAb protection. Thus, HVR1 may be protecting epitopes in a manner different from the generally accepted mechanism of direct steric shielding. Finally, our inability to induce high-level AR3A resistance in HCV retaining HVR1 indicates that the AR3A epitope has a high barrier to resistance, confirming the value of including this epitope in future vaccine candidates.

MATERIALS AND METHODS

Antibodies and reagents. Human monoclonal antibodies AR3A, AR4A, and AR5A against HCV envelope proteins were produced as described previously (21, 22). NS5A antibody 9E10 (66) was kindly provided by Charles Rice. E2 antibody H77.39 (67) was kindly provided by Michael S. Diamond. E2 antibody AP33 (68) was kindly provided by Arvind Patel. Plasmids with the JFH1 (genotype 2a) sequence (69) that has been further adapted to cell culture were previously described (46). Plasmids with the core-NS2 sequence from genotype 1a (H77) or genotype 2a (J6 or T9) as well as untranslated regions (UTRs) and NS3-NS5B from JFH1 with or (in the case of H77 and J6) without HVR1 were described previously (H77/JFH1$_{\Delta HVR1}$ has the HVR1 deletion adaptive mutations N476D and S733F) (34, 39, 66, 70), as was a further cell culture-adapted mutant, H77/JFH1$_{\Delta HVR1/N417D/N532D}$, which has two additional HVR1 deletion-adaptive mutations, N417D and N532D, in addition to N476D and S733F (24). Plasmids with point mutations were generated by conventional cloning techniques. The HCV sequence for each plasmid was confirmed by sequencing of the DNA preparation (Macrogen). Plasmids for viruses H77/JFH1$_{L438S/Q444R}$, H77/JFH1$_{\Delta HVR1/N417D/N532D/Q444R}$, and H77/JFH1$_{\Delta HVR1/N417D/N532D/L438S/Q444R}$ lost one uracil in the 3′ poly(U) tract during cloning. However, HCV poly(U) is able to tolerate single deletions without affecting virus replication (71, 72).

Cell culture of Huh7.5 cells. Huh7.5 cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco/Invitrogen Corporation, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin at 100 U/ml, and streptomycin at 100 μg/ml (Gibco/Invitrogen Corporation) in 5% CO_2 at 37°C. Cells were split every 48 to 72 h unless otherwise indicated.

Transfection of Huh7.5 cells. Huh7.5 cells were plated at 4×10^5 cells per well in 6-well plates 24 h prior to transfection. Plasmids were linearized by XbaI treatment (New England BioLabs). RNA was generated by T7-mediated *in vitro* transcription and was transfected into Huh7.5 cells using Lipofectamine 2000 (catalog number 11668-019; Invitrogen). At 6 h posttransfection, Huh7.5 cells were trypsinized and reseeded into 4 wells of 24-well plates at a cell density of 8×10^4 cells (for the 4 time points of the assay), along with plating in 6-well chamber slides for assessing the percentage of infected cells at the four time points, using the primary mouse anti-HCV NS5A 9E10 antibody and the secondary antibody Alexa 488 goat anti-mouse IgG(H+L) (Invitrogen), as described previously (73). Viral spread was monitored every 24 h along with harvesting of the virus supernatant up to 96 h posttransfection. Supernatants collected during experiments were sterile filtered and stored at −80°C. The virus titers were determined as described previously (74, 75).

AR3A escape assay. Huh7.5 cells were infected with HCVs of genotypes 1a and 2a with or without HVR1 at a multiplicity of infection (MOI) of 0.001 and incubated in 5% CO_2 at 37°C. Virus infection was monitored by immunostaining every 2 to 3 days as described above, and once the infection spread to 0.1% (H77/JFH1 and H77/JFH1$_{\Delta HVR1/N417D/N532D}$) or 5% to 10% (H77/JFH1$_{\Delta HVR1}$ and J6/JFH1$_{\Delta HVR1}$) of cells, cells were split into separate wells to be either treated with AR3A or left untreated as a control for viral spread. Control cultures consistently spread much faster (5 to 14 days) than AR3A-treated cultures. The cell supernatant was collected and filtered when cell infection reached 80% to 90% of the cells.

Antibody neutralization. We plated 6×10^3 Huh7.5 cells per well in poly-D-lysine-coated 96-well plates and incubated the cells for 24 h. The next day, 50 to 300 focus-forming units (FFU) of HCV were incubated in quadruplicates with a dilution series of monoclonal antibody and the relevant control antibody. Virus-antibody mixes along with eight replicates of virus only were incubated for 1 h at 37°C, added to Huh7.5 cells, and incubated for 4 h at 37°C. Subsequently, the cells were washed and incubated in fresh medium for a total infection time of 48 h. Cells were fixed and stained with 9E10 antibody, as described previously (73). The data were normalized with 8 replicates of virus only and analyzed using

three- or four-parameter curve fitting in GraphPad Prism (38). In all cases, analyses of neutralization data were repeated at least once.

Effect of temperature on neutralization (virus breathing). We plated 6×10^3 Huh7.5 cells per well in poly-D-lysine-coated 96-well plates and incubated the cells for 24 h. The next day, 600 FFU of HCV were preincubated in triplicates with a 3-fold dilution series of monoclonal antibody H77.39 (67) or AR3A (21). Virus-antibody mixes along with 6 replicates of virus only were incubated for 1 h at 4°C, 37°C, or 40°C. Antibody-virus mixtures were chilled, added to prechilled Huh7.5 cells, and spinoculated at 500 relative centrifugal force (RCF) for 2 h at 4°C. Subsequently, the cells were washed with cold phosphate-buffered saline (PBS) buffer, prechilled DMEM was added, the temperature was raised to 37°C, and cells were incubated for a total infection time of 48 h. Cells were fixed and stained with 9E10 antibody as described previously (73). The data were normalized with 6 replicates of virus only and analyzed using three-parameter curve fitting in GraphPad Prism (38).

Effect of pH on infectivity. Virus supernatants containing 20 mM HEPES were diluted 1:3 in citric acid buffer (15 mM citric acid, 150 mM NaCl) at pHs of 2, 4, and 6 to reach final pHs of 3, 5, and 7, respectively. The pH was determined by direct measurements with a standard pH meter. Virus-buffer mixtures were incubated for 15 min at 37°C, and 1/10 of the medium containing 150 mM HEPES was added. Finally, virus-buffer mixtures were diluted 1:5 with medium containing 20 mM HEPES to raise the pH to 7. The virus titers were determined as described previously (74, 75) and normalized to the pH 7 sample.

Virus stability assay. Virus supernatants were thawed and incubated at 4°C, 37°C, and 40°C for 0.5, 1, 2, 4, 8, 24, or 48 h prior to infectivity titration as described above. Virus titers were determined as described above.

Immunostaining of infected cells for evaluating AR3A binding. Huh7.5 cells were plated at 4×10^5 cells per well in 6-well plates 24 h prior to transfection. Huh7.5 cells were transfected as described above, immediately replated at 20,000 cells per well in 8-well slides, and incubated for an additional 48 h. Cells were fixed with paraformaldehyde at room temperature for 15 min, permeabilized using 0.5% digitonin, and stained using primary antibodies targeting E2 epitopes, AP33, and AR3A. We used a combination of Alexa 594 goat anti-mouse IgG(H+L) (Invitrogen) and Alexa 488 goat anti-human (Invitrogen) as secondary antibodies with a Hoechst 33342 (Molecular Probes) nucleic counterstain.

Flow cytometric analysis of AR3A binding. Huh7.5 cells were transfected with HCV RNA as described above, or HEK293T cells were transfected with J6 E1/E2 expression plasmids in a similar fashion. At 48 h posttransfection, cells were EDTA treated and subjected to two PBS washes between the following steps. Cells were fixed in 4% formaldehyde for 10 min and incubated for 1 h with primary antibodies AR3A (21) and AP33 (68), both at 1 μg/ml, This was followed by a 1-h incubation with the secondary antibodies Alexa Fluor 488 anti-human (ThermoFisher) and allophycocyanin (APC) anti-mouse (BioLegend). Cells were resuspended and analyzed on a Becton, Dickinson LSR Fortessa instrument equipped with 405-, 488-, and 640-nm lasers with suitable filter sets. Data were collected using FACSDiva 8 (Becton, Dickinson) and analyzed with FlowJo 10 software (FlowJo LLC). For data analysis, live cell populations were gated using front- and side-scatter dot plots and subsequently analyzed using AP33 (APC) and AR3A (Alexa Fluor 488) density plots. The population of double-positive cells was used to calculate the mean fluorescence intensity (MFI) and normalized AR3A fluorescence histograms.

Receptor blocking. We plated 6×10^3 Huh7.5 cells per well in poly-D-lysine-coated 96-well plates and incubated the cells for 24 h. The next day, Huh7.5 cells were incubated in four replicates with dilution series of previously used monoclonal antibody against CD81 or SR-BI (38) or with four replicates of the respective control antibodies at the highest concentrations used in the assay. Following a 1-h incubation, a volume of the virus stock corresponding to a readout of 50 to 100 FFU/well was added to the cell-antibody mix and incubated for 4 h at 37°C. Cells were washed, and we added fresh medium and incubated the cells for a total infection time of 48 h. Cells were fixed and stained with 9E10 antibody as described previously (73), and the number of FFU was counted as described above (74, 75). The data were normalized to 8 replicates of virus only and analyzed using three-parameter curve fitting in GraphPad Prism 7.02.

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REFERENCES

1. Bukh J. 2016. The history of hepatitis C virus (HCV): basic research reveals unique features in phylogeny, evolution and the viral life cycle with new perspectives for epidemic control. J Hepatol 65:S2–S21. https://doi.org/10.1016/j.jhep.2016.07.035.
2. World Health Organization. 2017. Hepatitis C. Fact sheet. World Health Organization, Geneva, Switzerland. https://www.who.int/news-room/fact-sheets/detail/hepatitis-c.
3. Pawlotsky JM, Feld JJ, Zeuzem S, Hoofnagle JH. 2015. From non-A, non-B hepatitis to hepatitis C virus cure. J Hepatol 62:S87–S99. https://doi.org/10.1016/j.jhep.2015.02.006.
4. Zoulim F, Liang TJ, Gerbes AL, Aghemo A, Deuffic-Burban S, Dusheiko G, Fried MW, Pol S, Rockstroh JK, Terrault NA, Wiktor S. 2015. Hepatitis C virus treatment in the real world: optimising treatment and access to therapies. Gut 64:1824–1833. https://doi.org/10.1136/gutjnl-2015-310421.
5. Walker CM. 2017. Designing an HCV vaccine: a unique convergence of prevention and therapy? Curr Opin Virol 23:113–119. https://doi.org/10.1016/j.coviro.2017.03.014.
6. Sarrazin C, Isakov V, Svarovskaia ES, Hedskog C, Martin R, Chodavarapu K, Brainard DM, Miller MD, Mo H, Molina JM, Sulkowski MS. 2017. Late relapse versus hepatitis C virus reinfection in patients with sustained virologic response after sofosbuvir-based therapies. Clin Infect Dis 64:44–52. https://doi.org/10.1093/cid/ciw676.
7. Bartenschlager R, Baumert TF, Bukh J, Houghton M, Lemon SM, Lindenbach BD, Lohmann V, Moradpour D, Pietschmann T, Rice CM, Thimme R, Wakita T. 2018. Critical challenges and emerging opportunities in hepatitis C virus research in an era of potent antiviral therapy: considerations for scientists and funding agencies. Virus Res 248:53–62. https://doi.org/10.1016/j.virusres.2018.02.016.
8. Robertson B, Myers G, Howard C, Brettin T, Bukh J, Gaschen B, Gojobori T, Maertens G, Mizokami M, Nainan O, Netesov S, Nishioka K, Shin-i T, Simmonds P, Smith D, Stuyver L, Weiner A. 1998. Classification, nomenclature, and database development for hepatitis C virus (HCV) and related viruses: proposals for standardization. International Committee on Virus Taxonomy. Arch Virol 143:2493–2503. https://doi.org/10.1007/s007050050479.
9. Smith DB, Bukh J, Kuiken C, Muerhoff AS, Rice CM, Stapleton JT, Simmonds P. 2014. Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment Web resource. Hepatology 59:318–327. https://doi.org/10.1002/hep.26744.
10. Fauvelle C, Colpitts CC, Keck ZY, Pierce BG, Foung SK, Baumert TF. 2016. Hepatitis C virus vaccine candidates inducing protective neutralizing antibodies. Expert Rev Vaccines 15:1535–1544. https://doi.org/10.1080/14760584.2016.1194759.
11. Pestka JM, Zeisel MB, Blaser E, Schurmann P, Bartosch B, Cosset FL, Patel AH, Meisel H, Baumert J, Viazov S, Rispeter K, Blum HE, Roggendorf M, Baumert TF. 2007. Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. Proc Natl Acad Sci U S A 104:6025–6030. https://doi.org/10.1073/pnas.0607026104.
12. Osburn WO, Snider AE, Wells BL, Latanich R, Bailey JR, Thomas DL, Cox AL, Ray SC. 2014. Clearance of hepatitis C infection is associated with the early appearance of broad neutralizing antibody responses. Hepatology 59:2140–2151. https://doi.org/10.1002/hep.27013.
13. Lavillette D, Morice Y, Germanidis G, Donot P, Soulier A, Pagkalos E, Sakellariou G, Intrator L, Bartosch B, Pawlotsky JM, Cosset FL. 2005. Human serum facilitates hepatitis C virus infection, and neutralizing responses inversely correlate with viral replication kinetics at the acute phase of hepatitis C virus infection. J Virol 79:6023–6034. https://doi.org/10.1128/JVI.79.10.6023-6034.2005.
14. de Jong YP, Dorner M, Mommersteeg MC, Xiao JW, Balazs AB, Robbins JB, Winer BY, Gerges S, Vega K, Labitt RN, Donovan BM, Giang E, Krishnan A, Chiriboga L, Charlton MR, Burton DR, Baltimore D, Law M, Rice CM, Ploss A. 2014. Broadly neutralizing antibodies abrogate established hepatitis C virus infection. Sci Transl Med 6:254ra129. https://doi.org/10.1126/scitranslmed.3009512.
15. Morin TJ, Broering TJ, Leav BA, Blair BM, Rowley KJ, Boucher EN, Wang Y, Cheslock PS, Knauber M, Olsen DB, Ludmerer SW, Szabo G, Finberg RW, Purcell RH, Lanford RE, Ambrosino DM, Molrine DC, Babcock GJ. 2012. Human monoclonal antibody HCV1 effectively prevents and treats HCV infection in chimpanzees. PLoS Pathog 8:e1002895. https://doi.org/10.1371/journal.ppat.1002895.
16. Prentoe J, Verhoye L, Velazquez MR, Buysschaert C, Farhoudi A, Wang R, Alter H, Meuleman P, Bukh J. 2016. HVR1-mediated antibody evasion of highly infectious in vivo adapted HCV in humanised mice. Gut 65:1988–1997. https://doi.org/10.1136/gutjnl-2015-310300.
17. Bukh J, Engle RE, Faulk K, Wang RY, Farci P, Alter HJ, Purcell RH. 2015. Immunoglobulin with high-titer in vitro cross-neutralizing hepatitis C virus antibodies passively protects chimpanzees from homologous, but not heterologous, challenge. J Virol 89:9128–9132. https://doi.org/10.1128/JVI.01194-15.
18. Vanwolleghem T, Bukh J, Meuleman P, Desombere I, Meunier JC, Alter H, Purcell RH, Leroux-Roels G. 2008. Polyclonal immunoglobulins from a chronic hepatitis C virus patient protect human liver-chimeric mice from infection with a homologous hepatitis C virus strain. Hepatology 47:1846–1855. https://doi.org/10.1002/hep.22244.
19. Meuleman P, Bukh J, Verhoye L, Farhoudi A, Vanwolleghem T, Wang RY, Desombere I, Alter H, Purcell RH, Leroux-Roels G. 2011. In vivo evaluation of the cross-genotype neutralizing activity of polyclonal antibodies against hepatitis C virus. Hepatology 53:755–762. https://doi.org/10.1002/hep.24171.
20. von Hahn T, Yoon JC, Alter H, Rice CM, Rehermann B, Balfe P, McKeating JA. 2007. Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection in vivo. Gastroenterology 132:667–678. https://doi.org/10.1053/j.gastro.2006.12.008.
21. Law M, Maruyama T, Lewis J, Giang E, Tarr AW, Stamataki Z, Gastaminza P, Chisari FV, Jones IM, Fox RI, Ball JK, McKeating JA, Kneteman NM, Burton DR. 2008. Broadly neutralizing antibodies protect against hepatitis C virus quasispecies challenge. Nat Med 14:25–27. https://doi.org/10.1038/nm1698.
22. Giang E, Dorner M, Prentoe JC, Dreux M, Evans MJ, Bukh J, Rice CM, Ploss A, Burton DR, Law M. 2012. Human broadly neutralizing antibodies to the envelope glycoprotein complex of hepatitis C virus. Proc Natl Acad Sci U S A 109:6205–6210. https://doi.org/10.1073/pnas.1114927109.
23. Prentoe J, Velazquez-Moctezuma R, Foung SK, Law M, Bukh J. 2016. Hypervariable region 1 shielding of hepatitis C virus is a main contributor to genotypic differences in neutralization sensitivity. Hepatology 64:1881–1892. https://doi.org/10.1002/hep.28705.
24. Velazquez-Moctezuma R, Law M, Bukh J, Prentoe J. 2017. Applying antibody-sensitive hypervariable region 1-deleted hepatitis C virus to the study of escape pathways of neutralizing human monoclonal antibody AR5A. PLoS Pathog 13:e1006214. https://doi.org/10.1371/journal.ppat.1006214.
25. Gal-Tanamy M, Keck ZY, Yi M, McKeating JA, Patel AH, Foung SK, Lemon SM. 2008. In vitro selection of a neutralization-resistant hepatitis C virus escape mutant. Proc Natl Acad Sci U S A 105:19450–19455. https://doi.org/10.1073/pnas.0809879105.
26. Keck ZY, Saha A, Xia J, Wang Y, Lau P, Krey T, Rey FA, Foung SK. 2011. Mapping a region of hepatitis C virus E2 that is responsible for escape from neutralizing antibodies and a core CD81-binding region that does not tolerate neutralization escape mutations. J Virol 85:10451–10463. https://doi.org/10.1128/JVI.05259-11.
27. Keck ZY, Angus AG, Wang W, Lau P, Wang Y, Gatherer D, Patel AH, Foung SK. 2014. Non-random escape pathways from a broadly neutralizing human monoclonal antibody map to a highly conserved region on the hepatitis C

virus E2 glycoprotein encompassing amino acids 412-423. PLoS Pathog 10:e1004297. https://doi.org/10.1371/journal.ppat.1004297.
28. Pantua H, Diao J, Ultsch M, Hazen M, Mathieu M, McCutcheon K, Takeda K, Date S, Cheung TK, Phung Q, Hass P, Arnott D, Hongo JA, Matthews DJ, Brown A, Patel AH, Kelley RF, Eigenbrot C, Kapadia SB. 2013. Glycan shifting on hepatitis C virus (HCV) E2 glycoprotein is a mechanism for escape from broadly neutralizing antibodies. J Mol Biol 425:1899–1914. https://doi.org/10.1016/j.jmb.2013.02.025.
29. Keck ZY, Olson O, Gal-Tanamy M, Xia J, Patel AH, Dreux M, Cosset FL, Lemon SM, Foung SK. 2008. A point mutation leading to hepatitis C virus escape from neutralization by a monoclonal antibody to a conserved conformational epitope. J Virol 82:6067–6072. https://doi.org/10.1128/JVI.00252-08.
30. Chung RT, Gordon FD, Curry MP, Schiano TD, Emre S, Corey K, Markmann JF, Hertl M, Pomposelli JJ, Pomfret EA, Florman S, Schilsky M, Broering TJ, Finberg RW, Szabo G, Zamore PD, Khettry U, Babcock GJ, Ambrosino DM, Leav B, Leney M, Smith HL, Molrine DC. 2013. Human monoclonal antibody MBL-HCV1 delays HCV viral rebound following liver transplantation: a randomized controlled study. Am J Transplant 13:1047–1054. https://doi.org/10.1111/ajt.12083.
31. Jensen TB, Gottwein JM, Scheel TK, Hoegh AM, Eugen-Olsen J, Bukh J. 2008. Highly efficient JFH1-based cell-culture system for hepatitis C virus genotype 5a: failure of homologous neutralizing-antibody treatment to control infection. J Infect Dis 198:1756–1765. https://doi.org/10.1086/593021.
32. Brimacombe CL, Grove J, Meredith LW, Hu K, Syder AJ, Flores MV, Timpe JM, Krieger SE, Baumert TF, Tellinghuisen TL, Wong-Staal F, Balfe P, McKeating JA. 2011. Neutralizing antibody-resistant hepatitis C virus cell-to-cell transmission. J Virol 85:596–605. https://doi.org/10.1128/JVI.01592-10.
33. Mathiesen CK, Prentoe J, Meredith LW, Jensen TB, Krarup H, McKeating JA, Gottwein JM, Bukh J. 2015. Adaptive mutations enhance assembly and cell-to-cell transmission of a high-titer hepatitis C virus genotype 5a core-NS2 JFH1-based recombinant. J Virol 89:7758–7775. https://doi.org/10.1128/JVI.00039-15.
34. Prentoe J, Jensen TB, Meuleman P, Serre SB, Scheel TK, Leroux-Roels G, Gottwein JM, Bukh J. 2011. Hypervariable region 1 differentially impacts viability of hepatitis C virus strains of genotypes 1 to 6 and impairs virus neutralization. J Virol 85:2224–2234. https://doi.org/10.1128/JVI.01594-10.
35. Forns X, Thimme R, Govindarajan S, Emerson SU, Purcell RH, Chisari FV, Bukh J. 2000. Hepatitis C virus lacking the hypervariable region 1 of the second envelope protein is infectious and causes acute resolving or persistent infection in chimpanzees. Proc Natl Acad Sci U S A 97:13318–13323. https://doi.org/10.1073/pnas.230453597.
36. Bankwitz D, Steinmann E, Bitzegeio J, Ciesek S, Friesland M, Herrmann E, Zeisel MB, Baumert TF, Keck ZY, Foung SK, Pecheur EI, Pietschmann T. 2010. Hepatitis C virus hypervariable region 1 modulates receptor interactions, conceals the CD81 binding site, and protects conserved neutralizing epitopes. J Virol 84:5751–5763. https://doi.org/10.1128/JVI.02200-09.
37. Scarselli E, Ansuini H, Cerino R, Roccasecca RM, Acali S, Filocamo G, Traboni C, Nicosia A, Cortese R, Vitelli A. 2002. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J 21:5017–5025. https://doi.org/10.1093/emboj/cdf529.
38. Prentoe J, Serre SB, Ramirez S, Nicosia A, Gottwein JM, Bukh J. 2014. Hypervariable region 1 deletion and required adaptive envelope mutations confer decreased dependency on scavenger receptor class B type I and low-density lipoprotein receptor for hepatitis C virus. J Virol 88:1725–1739. https://doi.org/10.1128/JVI.02017-13.
39. Scheel TK, Gottwein JM, Jensen TB, Prentoe JC, Hoegh AM, Alter HJ, Eugen-Olsen J, Bukh J. 2008. Development of JFH1-based cell culture systems for hepatitis C virus genotype 4a and evidence for cross-genotype neutralization. Proc Natl Acad Sci U S A 105:997–1002. https://doi.org/10.1073/pnas.0711044105.
40. Sabo MC, Luca VC, Ray SC, Bukh J, Fremont DH, Diamond MS. 2012. Hepatitis C virus epitope exposure and neutralization by antibodies is affected by time and temperature. Virology 422:174–184. https://doi.org/10.1016/j.virol.2011.10.023.
41. Lok SM, Kostyuchenko V, Nybakken GE, Holdaway HA, Battisti AJ, Sukupolvi-Petty S, Sedlak D, Fremont DH, Chipman PR, Roehrig JT, Diamond MS, Kuhn RJ, Rossmann MG. 2008. Binding of a neutralizing antibody to dengue virus alters the arrangement of surface glycoproteins. Nat Struct Mol Biol 15:312–317. https://doi.org/10.1038/nsmb.1382.
42. Dowd KA, Jost CA, Durbin AP, Whitehead SS, Pierson TC. 2011. A dynamic landscape for antibody binding modulates antibody-mediated neutralization of West Nile virus. PLoS Pathog 7:e1002111. https://doi.org/10.1371/journal.ppat.1002111.
43. Houghton M, Abrignani S. 2005. Prospects for a vaccine against the hepatitis C virus. Nature 436:961–966. https://doi.org/10.1038/nature04081.
44. Forns X, Bukh J, Purcell RH. 2002. The challenge of developing a vaccine against hepatitis C virus. J Hepatol 37:684–695. https://doi.org/10.1016/S0168-8278(02)00308-2.
45. Mikkelsen M, Bukh J. 2007. Current status of a hepatitis C vaccine: encouraging results but significant challenges ahead. Curr Infect Dis Rep 9:94–101. https://doi.org/10.1007/s11908-007-0003-6.
46. Carlsen TH, Pedersen J, Prentoe JC, Giang E, Keck ZY, Mikkelsen LS, Law M, Foung SK, Bukh J. 2014. Breadth of neutralization and synergy of clinically relevant human monoclonal antibodies against HCV genotypes 1a, 1b, 2a, 2b, 2c, and 3a. Hepatology 60:1551–1562. https://doi.org/10.1002/hep.27298.
47. Gopal R, Jackson K, Tzarum N, Kong L, Ettenger A, Guest J, Pfaff JM, Barnes T, Honda A, Giang E, Davidson E, Wilson IA, Doranz BJ, Law M. 2017. Probing the antigenicity of hepatitis C virus envelope glycoprotein complex by high-throughput mutagenesis. PLoS Pathog 13:e1006735. https://doi.org/10.1371/journal.ppat.1006735.
48. Owsianka AM, Timms JM, Tarr AW, Brown RJ, Hickling TP, Szwejk A, Bienkowska-Szewczyk K, Thomson BJ, Patel AH, Ball JK. 2006. Identification of conserved residues in the E2 envelope glycoprotein of the hepatitis C virus that are critical for CD81 binding. J Virol 80:8695–8704. https://doi.org/10.1128/JVI.00271-06.
49. Bailey JR, Wasilewski LN, Snider AE, El-Diwany R, Osburn WO, Keck Z, Foung SK, Ray SC. 2015. Naturally selected hepatitis C virus polymorphisms confer broad neutralizing antibody resistance. J Clin Invest 125:437–447. https://doi.org/10.1172/JCI78794.
50. Drummer HE, Boo I, Maerz AL, Poumbourios P. 2006. A conserved Gly436-Trp-Leu-Ala-Gly-Leu-Phe-Tyr motif in hepatitis C virus glycoprotein E2 is a determinant of CD81 binding and viral entry. J Virol 80:7844–7853. https://doi.org/10.1128/JVI.00029-06.
51. Grove J, Nielsen S, Zhong J, Bassendine MF, Drummer HE, Balfe P, McKeating JA. 2008. Identification of a residue in hepatitis C virus E2 glycoprotein that determines scavenger receptor BI and CD81 receptor dependency and sensitivity to neutralizing antibodies. J Virol 82:12020–12029. https://doi.org/10.1128/JVI.01569-08.
52. El-Diwany R, Cohen VJ, Mankowski MC, Wasilewski LN, Brady JK, Snider AE, Osburn WO, Murrell B, Ray SC, Bailey JR. 2017. Extra-epitopic hepatitis C virus polymorphisms confer resistance to broadly neutralizing antibodies by modulating binding to scavenger receptor B1. PLoS Pathog 13:e1006235. https://doi.org/10.1371/journal.ppat.1006235.
53. Li Q, Yafal AG, Lee YM, Hogle J, Chow M. 1994. Poliovirus neutralization by antibodies to internal epitopes of VP4 and VP1 results from reversible exposure of these sequences at physiological temperature. J Virol 68:3965–3970.
54. Dreux M, Pietschmann T, Granier C, Voisset C, Ricard-Blum S, Mangeot PE, Keck Z, Foung S, Vu-Dac N, Dubuisson J, Bartenschlager R, Lavillette D, Cosset FL. 2006. High density lipoprotein inhibits hepatitis C virus-neutralizing antibodies by stimulating cell entry via activation of the scavenger receptor BI. J Biol Chem 281:18285–18295. https://doi.org/10.1074/jbc.M602706200.
55. Bartosch B, Verney G, Dreux M, Donot P, Morice Y, Penin F, Pawlotsky JM, Lavillette D, Cosset FL. 2005. An interplay between hypervariable region 1 of the hepatitis C virus E2 glycoprotein, the scavenger receptor BI, and high-density lipoprotein promotes both enhancement of infection and protection against neutralizing antibodies. J Virol 79:8217–8229. https://doi.org/10.1128/JVI.79.13.8217-8229.2005.
56. Koutsoudakis G, Dragun J, Pérez-del-Pulgar S, Coto-Llerena M, Mensa L, Crespo G, González P, Navasa M, Forns X. 2012. Interplay between basic residues of hepatitis C virus glycoprotein E2 with viral receptors, neutralizing antibodies and lipoproteins. PLoS One 7:e52651. https://doi.org/10.1371/journal.pone.0052651.
57. Velazquez-Moctezuma R, Galli A, Law M, Bukh J, Prentoe J. 2019. Hepatitis C virus-escape studies for human monoclonal antibody AR4A reveal isolate-specific resistance and a high barrier to resistance. J Infect Dis 219(1):68–79. https://doi.org/10.1093/infdis/jiy481.
58. Gu J, Hardy J, Boo I, Vietheer P, McCaffrey K, Alhammad Y, Chopra A, Gaudieri S, Poumbourios P, Coulibaly F, Drummer HE. 2018. Escape of hepatitis C virus from epitope I neutralization increases sensitivity of

other neutralization epitopes. J Virol 92:e02066-17. https://doi.org/10.1128/JVI.02066-17.
59. He L, Cheng Y, Kong L, Azadnia P, Giang E, Kim J, Wood MR, Wilson IA, Law M, Zhu J. 2015. Approaching rational epitope vaccine design for hepatitis C virus with meta-server and multivalent scaffolding. Sci Rep 5:12501. https://doi.org/10.1038/srep12501.
60. Sandomenico A, Leonardi A, Berisio R, Sanguigno L, Foca G, Foca A, Ruggiero A, Doti N, Muscariello L, Barone D, Farina C, Owsianka A, Vitagliano L, Patel AH, Ruvo M. 2016. Generation and characterization of monoclonal antibodies against a cyclic variant of hepatitis C virus E2 epitope 412-422. J Virol 90:3745–3759. https://doi.org/10.1128/JVI.02397-15.
61. Pierce BG, Boucher EN, Piepenbrink KH, Ejemel M, Rapp CA, Thomas WD, Jr, Sundberg EJ, Weng Z, Wang Y. 2017. Structure-based design of hepatitis C virus vaccines that elicit neutralizing antibody responses to a conserved epitope. J Virol 91:e01032-17. https://doi.org/10.1128/JVI.01032-17.
62. Ren Y, Min YQ, Liu M, Chi L, Zhao P, Zhang XL. 2016. N-glycosylation-mutated HCV envelope glycoprotein complex enhances antigen-presenting activity and cellular and neutralizing antibody responses. Biochim Biophys Acta 1860:1764–1775. https://doi.org/10.1016/j.bbagen.2015.08.007.
63. Alhammad Y, Gu J, Boo I, Harrison D, McCaffrey K, Vietheer PT, Edwards S, Quinn C, Coulibaly F, Poumbourios P, Drummer HE. 2015. Monoclonal antibodies directed toward the hepatitis C virus glycoprotein E2 detect antigenic differences modulated by the N-terminal hypervariable region 1 (HVR1), HVR2, and intergenotypic variable region. J Virol 89:12245–12261. https://doi.org/10.1128/JVI.02070-15.
64. Vietheer PT, Boo I, Gu J, McCaffrey K, Edwards S, Owczarek C, Hardy MP, Fabri L, Center RJ, Poumbourios P, Drummer HE. 2017. The core domain of hepatitis C virus glycoprotein E2 generates potent cross-neutralizing antibodies in guinea pigs. Hepatology 65:1117–1131. https://doi.org/10.1002/hep.28989.
65. Burton DR, Hangartner L. 2016. Broadly neutralizing antibodies to HIV and their role in vaccine design. Annu Rev Immunol 34:635–659. https://doi.org/10.1146/annurev-immunol-041015-055515.
66. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM. 2005. Complete replication of hepatitis C virus in cell culture. Science 309:623–626. https://doi.org/10.1126/science.1114016.
67. Sabo MC, Luca VC, Prentoe J, Hopcraft SE, Blight KJ, Yi M, Lemon SM, Ball JK, Bukh J, Evans MJ, Fremont DH, Diamond MS. 2011. Neutralizing monoclonal antibodies against hepatitis C virus E2 protein bind discontinuous epitopes and inhibit infection at a postattachment step. J Virol 85:7005–7019. https://doi.org/10.1128/JVI.00586-11.
68. Owsianka A, Tarr AW, Juttla VS, Lavillette D, Bartosch B, Cosset FL, Ball JK, Patel AH. 2005. Monoclonal antibody AP33 defines a broadly neutralizing epitope on the hepatitis C virus E2 envelope glycoprotein. J Virol 79:11095–11104. https://doi.org/10.1128/JVI.79.17.11095-11104.2005.
69. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 11:791–796. https://doi.org/10.1038/nm1268.
70. Pedersen J, Carlsen TH, Prentoe J, Ramirez S, Jensen TB, Forns X, Alter H, Foung SK, Law M, Gottwein JM, Weis N, Bukh J. 2013. Neutralization resistance of hepatitis C virus can be overcome by recombinant human monoclonal antibodies. Hepatology 58:1587–1597.
71. Friebe P, Bartenschlager R. 2002. Genetic analysis of sequences in the 3′ nontranslated region of hepatitis C virus that are important for RNA replication. J Virol 76:5326–5338. https://doi.org/10.1128/JVI.76.11.5326-5338.2002.
72. You S, Rice CM. 2008. 3′ RNA elements in hepatitis C virus replication: kissing partners and long poly(U). J Virol 82:184–195. https://doi.org/10.1128/JVI.01796-07.
73. Gottwein JM, Scheel TK, Hoegh AM, Lademann JB, Eugen-Olsen J, Lisby G, Bukh J. 2007. Robust hepatitis C genotype 3a cell culture releasing adapted intergenotypic 3a/2a (S52/JFH1) viruses. Gastroenterology 133:1614–1626. https://doi.org/10.1053/j.gastro.2007.08.005.
74. Scheel TK, Gottwein JM, Carlsen TH, Li YP, Jensen TB, Spengler U, Weis N, Bukh J. 2011. Efficient culture adaptation of hepatitis C virus recombinants with genotype-specific core-NS2 by using previously identified mutations. J Virol 85:2891–2906. https://doi.org/10.1128/JVI.01605-10.
75. Gottwein JM, Scheel TK, Callendret B, Li YP, Eccleston HB, Engle RE, Govindarajan S, Satterfield W, Purcell RH, Walker CM, Bukh J. 2010. Novel infectious cDNA clones of hepatitis C virus genotype 3a (strain S52) and 4a (strain ED43): genetic analyses and in vivo pathogenesis studies. J Virol 84:5277–5293. https://doi.org/10.1128/JVI.02667-09.